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THE ROLE OF VIF IN OVERCOMING THE APOBEC3G
BLOCK TO HIV-1 REPLICATION

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A thesis submitted in fulfilment of the requirements for the
degree of Doctor of Philosophy at the University of Warwick

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ABBREVIATIONS

A	Alanine
A3G	APOBEC3G
AD	Activation Domain
AIDS	Acquired Immune Deficiency Syndrome
APOBEC	Apolipoprotein B mRNA-editing Enzyme-catalytic polypeptide
BD	DNA-binding domain
C	Cysteine
CA	Capsid
CAT	Chloramphenicol Acetyl Transferase
CD	Catalytic domain
CDC	Centre for Disease Control
cDNA	Complementary DNA
cPPT	Central Polypurine Tract
CTL	Cytotoxic T- lymphocytes
CypA	Cyclophilin A
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
E	Glutamic Acid
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
FCS	Foetal Calf Serum
FDA	Food and Drug Administration
g	Grams

GFP	Green Fluorescent Protein
GPCR	G-protein coupled receptor
GST	Glutathione-S-Transferase
HIV	Human Immunodeficiency Virus
HMM	High molecular mass
HPA	Health Protection Agency
HTLV	Human T-lymphotropic Virus
Ig	Immunoglobulin
IN	Integrase
K	Lysine
Kb	Kilobases
kDA	Kilo Daltons
LAV	Lymphadenopathy-associated Virus
LB	Luria Bertani
LMM	Low molecular mass
LTR	Long terminal repeat
MA	Matrix
mA	Milli Ampere
M	Mole
MHC	Major Histocompatibility Complex
MHR	Major Homology Region
ml	Milli Litre
mM	Milli Molar
mRNA	Messenger RNA
mRNP	Messenger Ribonucleoprotein Complex

NC	Nucleocapsid
ng	Nano gram
NLS	Nuclear localisation signal
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCD	Pseudocatalytic domain
PIC	Pre-integration complex
PPT	Polypurine tract
PR	Protease
R	Arginine
RNA	Ribonucleic acid
RRE	Rev response element
RT	Reverse Transcriptase
RTN	Reverse Transcription
SAP	Shrimp Alkaline Phosphatase
SDS	Sodium dodecyl sulphate
SOCS	Suppressor of cytokine signalling
SPR	Surface Plasmon Resonance
sssDNA	Strand strong stop DNA
TAR	Transactivation response element
TBE	Tris-Boric acid-EDTA
TCA	Trichloroacetic acid

tRNA	Transfer RNA
U	Units
UAS	Upstream activation sequence
UTR	Untranslated region
V	Volts
v/v	Volume for volume
Vif	Virus Infectivity Factor
VLP	Virus-like particle
w/v	Weight for volume
⁰ C	Celsius
μl	Micro litre

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DECLARATION

All results presented in this thesis were obtained by the author unless specifically acknowledged, and no part of this thesis has been previously presented in application for a degree.

All sources of information and materials are indicated in the text.

SUMMARY

This project focuses on the Virus Infectivity Factor protein of HIV-1 and its relief of the block to virus replication exerted by the APOBEC3G component of the innate immune response.

Virus Infectivity Factor (vif) is an accessory gene of HIV, deletion of which leads to large drops in virus infectivity. This decrease in infectivity was found to be due to APOBEC3G, an inhibitor of HIV replication which is constitutively expressed in peripheral blood mononuclear cells (PBMCs), the natural host cells for HIV in humans. Vif is indispensable to block the inhibitory effects of APOBEC3G thereby allowing normal viral replication to continue inside the host. This recognition of the critical role played by Vif in the viral replication cycle has centred studies on characterising the interactions of Vif with both APOBEC3G as well as other virus encoded proteins.

Stimulation of proteasomal degradation of APOBEC3G is currently believed to be the primary anti-APOBEC3G effect induced by Vif. However recent reports, particularly those showing that Vif remains able to block the inhibitory actions of degradation resistant APOBEC3G, question the validity of this hypothesis. The recognition that both APOBEC3G and Vif become incorporated into HIV particles through an interaction with the precursor of the virion structural proteins, Pr55^{GAG} has raised the possibility that they may compete with each other for this incorporation. Using techniques such as mammalian two-hybrid assays, sucrose gradient analysis of GAG virus-like particles (VLPs) and confocal imaging, the interactions of these proteins with Pr55^{GAG} have been analyzed and the results obtained indicate that Vif competes with APOBEC3G for Pr55^{GAG} binding leading to its displacement and exclusion from the budding HIV virions. This potentially important pathway for Vif activity and its significance in the development of novel antiretroviral drugs in the future will be discussed.

CHAPTER 1

General Introduction

1.1 History of HIV

The origin of HIV was from non-human primates in sub-saharan Africa. The virus is believed to have crossed the species barrier and infected humans sometime in the late 19th or early 20th century (Worobey et al, 2008). The current HIV epidemic is known to have started in the late 1970s. Prior to this time, nothing was known about the virus due to the silent spread and slow evolution of infection.

The first indications of this new infection came in 1981 in Los Angeles and New York when there was a sudden unexplained increase in cases of two very rare diseases: Kaposi's sarcoma and Pneumocystis Carinii pneumonia especially among homosexuals and intravenous drug users (Gottlieb et al, 1981; Hymes et al, 1981; Masur et al, 1981). The affected individuals appeared to have become immune-compromised thus rendering them susceptible to relatively avirulent organisms and malignancies. The term Acquired Immune Deficiency Syndrome (AIDS) was first coined by the CDC, Atlanta in 1982 to explain the occurrence of these uncommon diseases in a cluster in high-risk groups.

In 1983, Luc Montagnier's team at the Pasteur Institute in France isolated a virus from an African patient with persistent generalised lymphadenopathy as the causative agent of AIDS and named it Lymphadenopathy associated Virus (LAV) (Barré-Sinoussi et al, 1983). Later in 1984, Robert Gallo in the U.S confirmed the isolation of a retrovirus from AIDS patients and called it Human T-Lymphotropic Virus III (HTLV-III) (Popovic et al, 1984). The International Committee on Taxonomy of Viruses gave a new generic name Human Immunodeficiency Virus (HIV) for these viruses in 1986 (Coffin et al).

The first blood test for AIDS became available in 1985 and was based on the detection of antibodies to the virus in patient sera. Up until that point, the infection could only be diagnosed on the basis of characteristic opportunistic infections and malignancies. Following the availability of laboratory based diagnosis of infection, it quickly became clear that these terminal cases constituted only a minuscule proportion of the people infected with the virus. Following its recognition, the HIV epidemic has continued to rise and it has become one of the greatest threats to human health and well being. At the same time, there has been a lot of effort to study the virus, its mode of infection, its prevention and options for treatment. These efforts have led to a huge database of information which has increased our understanding both about the virus and the disease it causes.

1.2 Classification

The Human Immunodeficiency Virus (HIV) belongs to the Genus Lentivirus, which is a part of the Family Retroviridae. It is classified in Class VI of The Baltimore Classification of Viruses, it being a single stranded, positive sense RNA virus with an envelope that produces a DNA intermediate in its replication cycle. Two types of HIV are known, HIV-1 which originated in equatorial Africa and is closely related to a virus infecting Chimpanzees and HIV-2 which is confined to West Africa and is similar to a virus infecting Sooty Mangabey, an Old World Monkey (Gao et al, 1999; Reeves & Doms, 2002). HIV-1 is highly transmissible and is the major cause of the global HIV pandemic while HIV-2 has much lower transmissibility and is largely confined to West Africa.

1.3 Epidemiology

HIV was unknown less than 40 years ago. So dramatic has been the spread of the epidemic that it has led to almost 25 million deaths worldwide since it started in the late 1970s. Currently there are almost 33 million people worldwide who are living with HIV.

According to the latest UNAIDS report published in 2008, the annual number of new infections declined from 3.0 million in 2001 to 2.7 million in 2007. On the other hand, almost 2.0 million people died of AIDS in 2007 as compared to 1.7 million in 2001.

Globally, the epidemic has now stabilised but the number of new infections and AIDS related deaths are still unacceptably high. The number of people living with HIV is also steadily rising. This is due to a continued increase in the number of new infections particularly in economically less developed regions accompanied by the life-prolonging effects of anti-retroviral therapy.

The HIV Epidemic has been disproportionately concentrated in sub-saharan Africa with 35% of the new HIV infections and 38% of AIDS deaths in 2007 occurring in that region (Fig.1.1). Almost 67% of the people living with HIV are also concentrated in sub-saharan Africa (2008 UNAIDS report).

In the UK, an estimated 83000 people were living with HIV at the end of 2008 with almost one-third of these people being unaware of their infection. During 2008, there were 7298 new cases of HIV diagnosed in the UK (2009 HPA report).

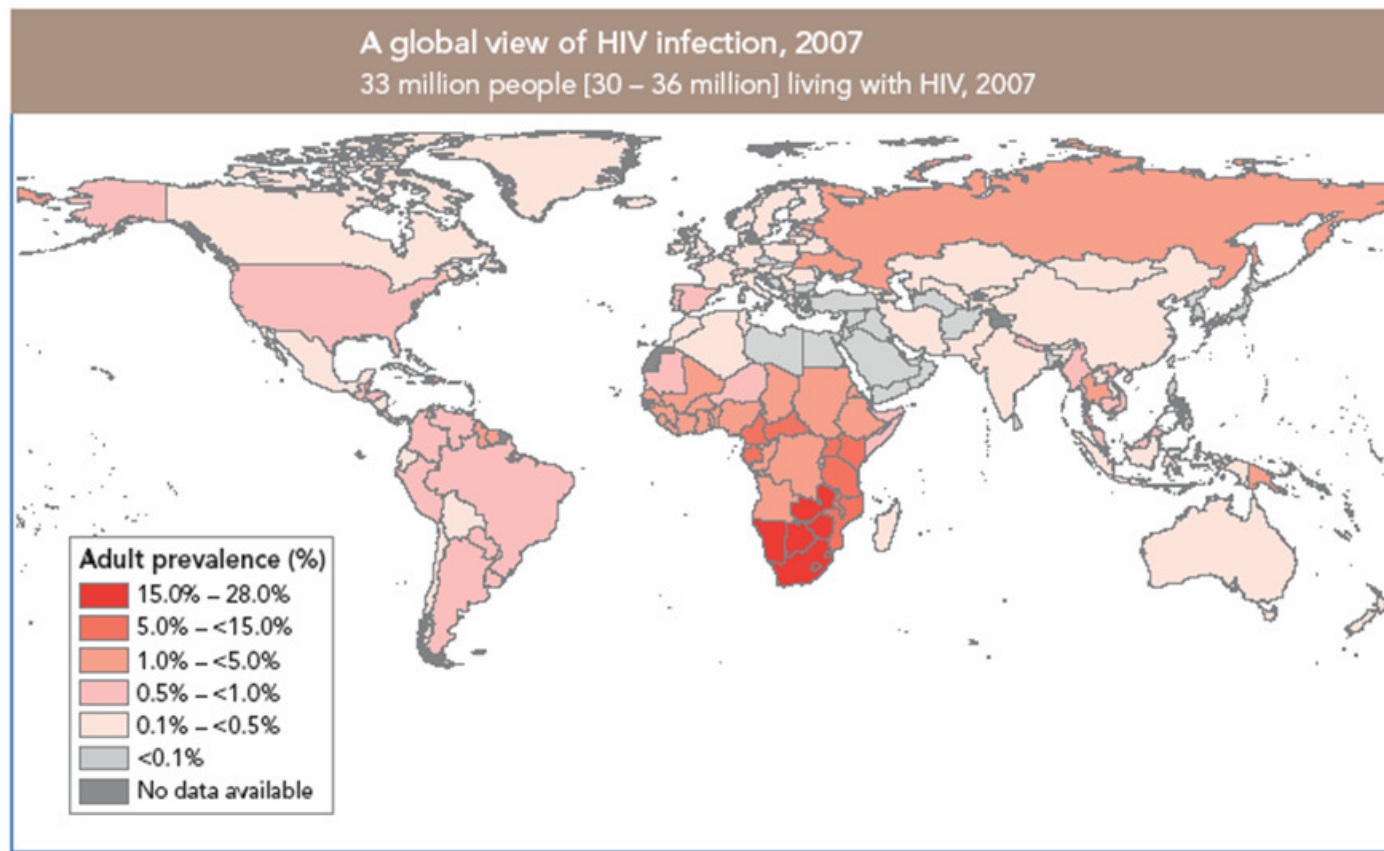


Figure 1.1 Status of the global HIV epidemic in 2007, 2008 UNAIDS report: The HIV epidemic is disproportionately concentrated in southern Africa with the highest adult prevalence in that region (15-28%).

1.4 Routes of transmission

HIV is transmitted between humans in three main ways:

1.4.1 Blood or blood products

Cause of infections in intravenous drug users and people receiving blood transfusions.

1.4.2 Mother to child transmission

This mode of infection is important in an HIV infected pregnant woman. Transmission of the virus occurs primarily during child birth or in the immediate post-natal period.

1.4.3 Sexual intercourse

Unprotected sexual intercourse is the most common route of infection.

1.5 Antigenic variation and diversity of HIV

HIV is a highly mutable virus. There are a lot of antigenic differences observed in the HIV genome from different isolates. Not only are HIV isolates obtained from different geographical areas different but isolates obtained from different individuals in the same region or more significantly isolates obtained from different sites of the same individual at the same time are also different (Ananthanarayan & Paniker, 2000). These variations are believed to occur due to the nature of the reverse transcription process which lacks an error correction mechanism.

As described in section 1.2, HIV has been classified into two types i.e. HIV-1 and HIV-2 based on molecular and antigenic differences. HIV-1 and HIV-2 were transferred to humans from two distinct simian viruses and hence have low sequence identity.

HIV-1 strains belonging to group M have been classified into genetically distinct subtypes or clades designated from A to K and these cause a majority of HIV-1 infections worldwide. Some isolates from West Central Africa don't fall under group M and are designated as group O (Outlier). In addition, some isolates found in Cameroon don't fall in either group M or O and hence have been designated as group N (New). Recently a new virus strain derived from gorillas has also been found in Cameroon which doesn't belong to any of these three groups. This strain has been designated as belonging to a new group P (Plantier et al, 2009).

The HIV-1 subtypes show differentiated geographical distribution. Subtype A is the most common and is prevalent worldwide. Subtype B predominates in the Americas and Europe. Subtypes A, C and D are common in Africa while subtypes E, C and B are common in Asia, with subtype C being the most prevalent clade in India and China.

1.6 Evolution of HIV infection

The natural course of HIV infection can be divided into the following stages (Fig. 1.2) (Ananthanarayan & Paniker, 2000):

1.6.1 Acute HIV infection

In the 3-6 week period following initial HIV infection, about 50% of the infected individuals experience low grade fever, headache, malaise and lymphadenopathy. These symptoms resolve spontaneously after a relatively short time (< 2-3 weeks). In some people, this stage may be completely absent. Tests for HIV antibodies are generally negative initially but may become positive later in this stage. Hence this stage is also referred to as 'seroconversion illness'. The symptoms during this period occur as a result of both the immune response as well as virus multiplication.

1.6.2 Latent infection

Every infected individual whether he/she goes through the first phase or not experiences the second stage of HIV infection. This stage which comprises symptomless chronic infection may last up to several years. In this phase, viral multiplication proceeds normally but due to the immune response mounted against the infection the viral load is kept under control thus preventing any clinical manifestations. However, the immune system is not capable of clearing the infection completely resulting in a simultaneous gradual decrease in CD4+ T cell count.

1.6.3 Persistent generalised lymphadenopathy

This is defined as the presence of enlarged lymph nodes, at least 1 cm in diameter, in two or more non-contiguous extrainguinal areas which persist for at least 3 months in the absence of any other illness or medication. This stage may progress to full blown AIDS.

1.6.4 AIDS related complex (ARC)

During this phase, infected individuals suffer from various symptoms such as fatigue, loss of weight, diarrhoea and unexplained fever. Due to their immunocompromised state, minor opportunistic infections such as herpes zoster, oral candidiasis, salmonellosis and tuberculosis are fairly common. Many of the ARC patients progress to full blown AIDS in a few months.

1.6.5 AIDS

This stage represents the end stage of the illness and involves the irreversible breakdown of the immune system. As the immune system becomes weak, opportunistic infections and malignancies become more common. Patients are vulnerable to infections such as *Pneumocystis carinii*, *Mycobacterium tuberculosis*, *Salmonella* and *Cytomegalovirus* and malignancies like Kaposi's sarcoma and lymphomas. The severity of AIDS depends on the type of infection or malignancy present.

1.7 Diagnostic tests for HIV

Different types of diagnostic tests are employed for the detection of HIV infection in humans (Ananthanarayan & Paniker, 2000). They are as follows:

1.7.1 Detection of virus antigen

The p24 antigen capture assay (ELISA) is the most common technique used for the detection of HIV p24 proteins in patient serum. If the patient gets infected with a large dose of infectious material, the antigen becomes positive as early as 2-3 weeks

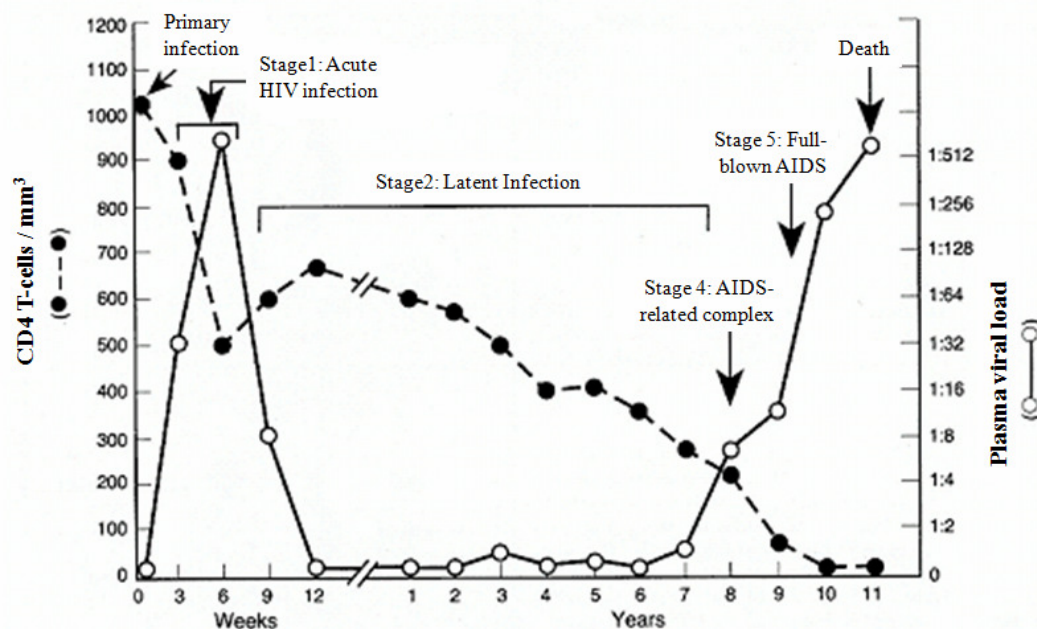


Figure 1.2 Clinical course of an untreated HIV infection: As the CD4 T-cell count decreases in the acute stage of HIV infection, the plasma viral load increases. In the latent phase of HIV which can last as long as 10-15 years, the plasma viral load remains low as the virus remains dormant. During the corresponding period, the CD4 count starts dropping gradually. In the AIDS-related complex stage, the CD4 count becomes very low and viral load starts to increase again as the immune system can't control the infection. A number of opportunistic infections may occur during this period due to the immunocompromised state. Patients in this stage of infection usually proceed to full-blown AIDS in a couple of months. AIDS represents the end stage of the infection and complete breakdown of the immune system. (Figure modified from Pantaleo G et al ,1993).

after exposure. However if the infective dose is small, it may take up to 4-6 weeks for the appearance of p24 in the patient's serum. The p24 antigen is positive in the beginning of infection, becomes negative in the latent phase and resurfaces again at the end-stage of disease.

1.7.2 Detection of viral load

The viral load after infection mirrors p24 antigen levels. It is high immediately following infection in the first stage of the disease. Later, it becomes low in the latent stage and rises again in the end-stage of disease. The diagnostic technique employs incubation of the patient's lymphocytes with uninfected lymphocytes and Interleukin-2. The presence of HIV is detected by reverse transcriptase activity and positive viral antigens. However, this test can't be used for large scale diagnostic screening as it gives many false negative results. In addition, due to the risks associated with working with infectious virus, this test can only be employed in labs with suitable containment facilities.

1.7.3 Polymerase chain reaction

This test is the gold standard for the diagnosis of HIV at all stages of the infection. However, it is too expensive to be used routinely as a screening test for HIV infection. In this technique, the patient's lymphocytes are lysed and proviral DNA/RNA is amplified using primers specific for relatively constant regions of the HIV genome. The amplified products are characterised using nucleic acid hybridisation. It is a very sensitive test and if done with proper controls, the proviral DNA can be detected at a frequency of 1 copy per 10,000 cells.

1.7.4 Detection of the virus antibody in patient's serum

Antibody detection is the simplest and most commonly used diagnostic method for detection of HIV infection. However, it may take up to several weeks for antibodies to develop to detectable levels in serum. This period during which the antibody titres are negative but the person is infectious is called the 'window period'. The tests employed for detecting antibodies against HIV can be divided into two types:

1.7.4.1 Screening antibody capture assay (ELISA)

Antigens representing all groups and subtypes of HIV-1 and HIV-2 obtained from HIV grown in T-lymphocyte cell lines or by recombinant techniques are coated onto the wells of ELISA micro titre plates. The patient's serum is incubated with the antigens and if the serum is positive for HIV antibodies, it can be detected colorimetrically using anti-human immunoglobulin as the secondary antibody. This test is highly sensitive but not so specific and can yield many false positives. However, it is easy to perform and is used on a large-scale for screening patients.

1.7.4.2 Confirmatory assay (Western blot)

In this test, HIV proteins are separated using SDS-PAGE and blotted onto nitrocellulose membranes. The blots are probed by test sera and enzyme-conjugated anti-human immunoglobulin is used as a secondary antibody. The western blot is a very specific test and is usually used to confirm results obtained in a screening antibody capture ELISA.

1.8 Treatment of HIV infection

The treatment of HIV infection involves a three-pronged strategy:

1.8.1 Prevention and prophylaxis of opportunistic infections and malignancies

As the immune system of an HIV infected individual becomes compromised, the person becomes susceptible to numerous opportunistic infections and malignancies. Diagnosing them without delay and initiating proper treatment of these infections is important in improving the health of an individual.

1.8.2 Specific anti-HIV drugs

In addition to the treatment of opportunistic diseases, it is of utmost importance to keep the HIV infection itself under control. Hence regular monitoring of the CD4+ T cell count and viral load (viral RNA levels) of the patient is recommended. If the CD4+ T cell count drops below $350/\text{mm}^3$ or viral load increases, treatment with specific anti-retroviral drugs is initiated. There are more than 20 Food and Drugs Administration (FDA) approved drugs currently targeting HIV and they can be divided into 5 groups:

1.8.2.1 Nucleoside reverse transcriptase inhibitors (NRTIs): e.g. Zidovudine (AZT), Lamivudine (3TC), Didanosine (ddI).

Mechanism of action: NRTIs as their name suggests are nucleoside analogues. AZT is a thymidine analogue, 3TC is a cytidine analogue and ddI is an adenosine analogue. They get incorporated into viral cDNA by mimicking nucleotides and compete with the deoxyribonucleotides within the cells (Perno et al, 1988; Sarafianos et al, 2009). The only structural difference is that they lack a 3'OH group on the deoxyribose sugar. This leads to the 5'-3'phosphodiester bond needed to extend the

DNA chain not being formed. This process is called chain termination. However, before they can prematurely terminate the formation of viral cDNA, nucleoside analogues need to be activated to nucleotides by phosphorylation (Furman et al, 1986). This step is carried out within the cell by cellular kinases. Thus NRTIs act during the reverse transcription process and prematurely terminate the formation of viral cDNA thereby inhibiting HIV replication.

1.8.2.2 Non-nucleoside reverse transcriptase inhibitors (NNRTIs): e.g. Delavirdine (DLV), Efavirenz (EFV), Nevirapine (NVP).

Mechanism of action: NNRTIs are chemically different from NRTIs and do not require cellular kinases to activate them. All NNRTIs though chemically different interact at the same site on HIV-1 reverse transcriptase. They bind to a region of the p66 subunit of the HIV-1 RT p66/p51 heterodimer called the NNRTI binding pocket thus inhibiting the enzyme from completing reverse transcription. It is important to note here that NNRTIs cannot bind to HIV-2 RT and hence can't inhibit HIV-2 replication (De Clercq, 1998).

1.8.2.3 Protease inhibitors (PI): e.g. Indinavir (IDV), Nelfinavir (NFV), Amprenavir (APV).

Mechanism of action: The HIV-1 protease cleaves the polyprotein precursors Pr55^{GAG} and Pr160^{GAG-POL} into smaller proteins resulting in the reorganisation of the virion structure by a process called maturation. Maturation of the virus particle during or just after budding is critical for subsequent virus infectivity. PIs are synthetic analogues of the phenylalanine-proline cleavage site on Pr55^{GAG} (Debouck, 1992). They act on the viral protease and prevent the cleavage of the polyprotein precursor thereby blocking the process of maturation and infectivity of nascent

virions. Thus the primary action of PIs is to prevent subsequent waves of infection and they have no effect on the cells harbouring integrated proviral DNA (Flexner, 1998).

1.8.2.4 Fusion or entry inhibitors: e.g. Enfuvirtide (T-20), Maraviroc (MVC).

Mechanism of action: There are currently two approved entry inhibitors with different targets but each affecting the process of viral entry into the cell.

a) Maraviroc – It is a selective CCR5 antagonist and small-molecule inhibitor which inhibits HIV-1 gp120 binding to CCR5, thus preventing gp160-CCR5-mediated cell-cell fusion and therefore inhibits HIV entry into cells (Dorr et al, 2005). It is an allosteric inhibitor which interacts with the hydrophobic pocket in the transmembrane helices of CCR5 and changes the conformation of the extracellular loops thus disrupting the gp120 binding site (Tilton & Doms, 2010).

b) Enfuvirtide – It is a 36-mer oligopeptide whose sequence corresponds to the HR2 region of gp41. It interacts with the HR-1 trimeric region of gp41 and prevents the association between HR2 and HR1. The association between HR1 and HR2 is essential to proximate the viral and cellular membrane and form a viral pore for entry into the cell cytoplasm (discussed in greater detail in sections 1.10.1.3 and 1.11.1). Enfuvirtide inhibits this final step of viral entry and thus prevents infection (Tilton & Doms, 2010).

1.8.2.5 Integrase inhibitors: e.g. Raltegravir (RAL).

Mechanism of action: Integration of the HIV DNA into the host chromosomal DNA is a multi-step process catalysed by the viral enzyme integrase. It involves interaction of the enzyme with the viral DNA, formation of a stable pre-integration complex,

transfer of the complex from the cytoplasm to the nucleus and finally the integration of the viral DNA into the host genome. Integrase inhibitors act on the active sites within the integrase and prevent the viral strand transfer into the host thus preventing the cells from forming an integrated provirus. As the viral DNA can't integrate, it is targeted by the cellular DNA repair mechanisms and rendered inactive (Cocohoba & Dong, 2008).

The treatment strategy used with these drugs is called Highly Active Anti-retroviral Therapy (HAART). The principle behind this strategy is administering a combination of 2 or more drugs concurrently can significantly delay the development of viral resistance. If the same drugs were to be given individually and sequentially, the onset of resistance would be much quicker. Although HAART is not a cure for HIV, it can significantly increase the life span of people infected with the disease.

1.8.3 General health management of the patient

When a patient is diagnosed with HIV, it becomes very important to remove some of the misconceptions about the disease. Hence counselling is an important part of treatment. In addition, nutritional guidance and treatment compliance are two significant arms that need to be explained in detail to the patient.

Thus regular health check-ups, adhering strictly to treatment regimens and proper nutrition can help the patient control HIV infection for many years enabling him/her to lead an almost normal life.

1.9 Structure of the HIV virion

The HIV particle is an approximately 120nm spherical particle with an outer envelope made up of lipid (Fig. 1.3). 72 spikes made up of proteins gp120 and gp41 project from the envelope. Inside of the envelope, lies the layer of matrix protein, also called p17. Lining the spherical matrix protein from inside is the toroidally shaped nucleocapsid, whose predominant constituent is p24, the serologic marker of HIV replication. The nucleocapsid also contains three enzymes Reverse Transcriptase (RT), Protease (PR) and Integrase (IN) which are essential for viral replication (Gelderblom et al, 1988; Haseltine, 1991). In addition it contains two identical functional strands of viral genetic material which is single stranded positive sense RNA. Hence retroviruses are unusual as the sole examples of genetically diploid viruses.

1.10 Genes and gene products of HIV

HIV genome consists of 9181 bases of linear single stranded RNA. As shown in figure 1.4, the integrated provirus encodes 9 proteins. At either end are Long Terminal Repeats (LTR) involved in the initiation of transcription and viral replication.

The genes of HIV can be classified as follows:

1.10.1 Structural genes / viral proteins

1.10.1.1 Gag

The Gag gene of HIV encodes a polyprotein precursor, Pr55^{GAG} (named on the basis of its molecular weight, 55 kDa) (Fig. 1.4). Pr55^{GAG} is on its own capable of self-

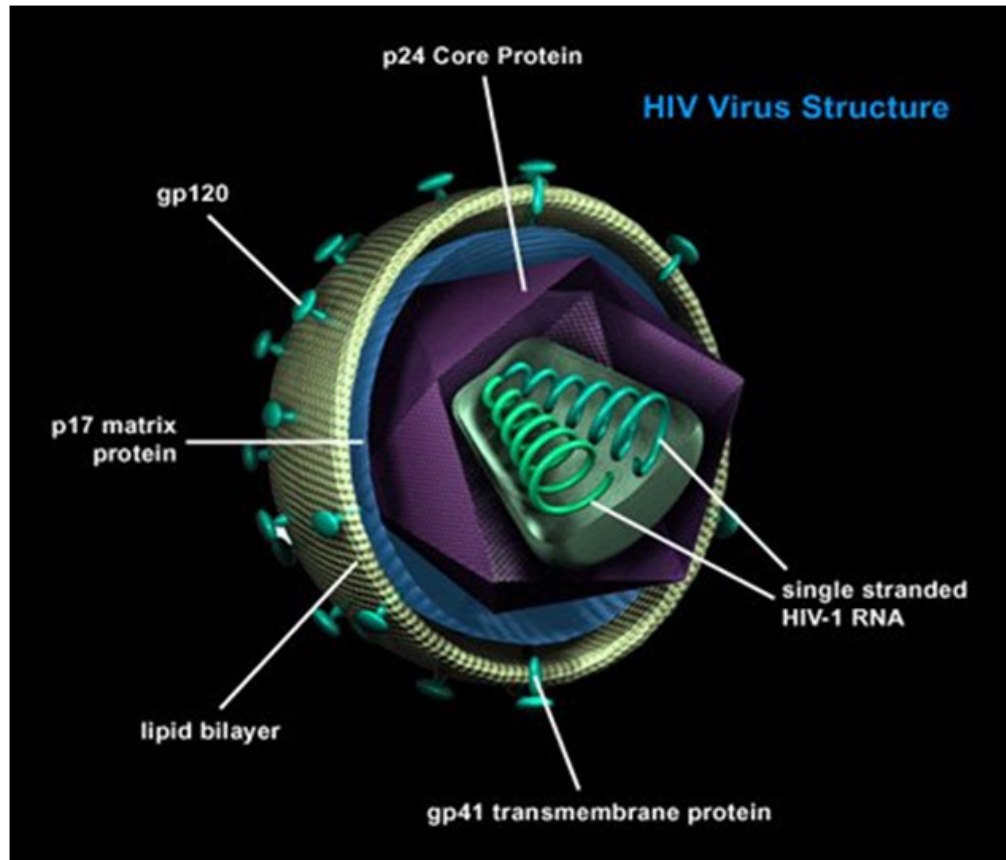


Figure 1.3 Diagram to illustrate the structure of the HIV virion: The outermost layer is made up of lipids. From this layer project 72 spikes made up of the envelope glycoproteins gp120 and gp41. The envelope is lined from the inside by the matrix protein (p17) layer. The innermost toroidal core is made up predominantly of the capsid protein (p24). The capsid encloses two identical strands of viral genetic material, single stranded positive sense RNA along with the viral enzymes Reverse Transcriptase, Protease and Integrase. (Courtesy: HIV InSite)

assembly to generate immature Gag virus-like particles even in the absence of other viral proteins or genomic RNA (Gheysen et al, 1989; Karacostas et al, 1989). The structural proteins of HIV have to perform two very contrasting functions if HIV is to replicate successfully in its host cells. On the one hand, they have to disassemble into small proteins and help direct the viral genetic material to the nucleus during the post-entry phase while on the other hand during the post-integration phase they have to form ordered multimerised structures capable of localising to the cytoplasmic membrane, initiating assembly of the progeny virus particles and their subsequent budding from the cell surface. These dual functions are achieved by Pr55^{GAG} first performing the assembly function and later being cleaved into subcomponent proteins by the viral enzyme protease (Freed, 1998).

During or shortly after budding Pr55^{GAG} is cleaved into matrix (MA or p17), capsid (CA or p24), nucleocapsid (NC or p7) and p6 (Henderson et al, 1992). Two spacer peptides p2 and p1 are also generated from the cleavage process (Sheng & Ericksonviitanen, 1994). These latter two small peptides assist in the ordered cleavage and maturation of virus particles (Pettit et al, 1994). The process of cleavage of Pr55^{GAG} into different fragments is called maturation as it results in many morphological changes in the newly released virus particles. MA remains attached to the inner surface of the viral membrane while CA forms a conical core around the NC-viral RNA complex (Gelderblom et al, 1988). The end result of maturation is the formation of fully infectious HIV particles capable of invading other cells. The functions of the individual fragments generated as a result of cleavage are discussed below:

MA is formed from the cleavage of the N-terminal region of Pr55^{GAG}. The N-myristoylated MA protein has been implicated in directing Pr55^{GAG} to the plasma

membrane and initiating particle assembly (Bryant & Ratner, 1990; Gheysen et al, 1989; Gottlinger et al, 1989; Hermida-Matsumoto & Resh, 2000). It also plays a role in Env incorporation into the budding virus particles which is demonstrated by the fact that multiple amino acid substitutions (Dorfman et al, 1994b) and single residue mutations (Freed & Martin, 1995b) in HIV-1 MA block Env incorporation into the HIV particles. In the post-entry stage of the viral life cycle MA has been found to be a part of the pre-integration complex which is responsible for transporting viral DNA produced by reverse transcription of the viral genome to the nucleus (Bukrinsky et al, 1993b; Gallay et al, 1995; Miller et al, 1997).

CA or p24 is the second sequential product of Pr55^{GAG} cleavage. The conical shell which protects the viral RNA is made up predominantly of CA. The C terminal domain of CA contains a highly conserved sequence, the major homology region (MHR), which has been shown to be involved in Gag oligomerisation (Dorfman et al, 1994a; Zhang et al, 1996). CA has also been shown to be involved in the incorporation of Gag-Pol precursor into the budding HIV virions thus enabling RT, PR and IN to be incorporated into the virion (Smith et al, 1993; Srinivasakumar et al, 1995). The N-terminal domain of CA has been reported to be essential in the specific incorporation of Cyclophilin A (Cyp A) in the virion (Franke et al, 1994; Thali et al, 1994). Virions lacking Cyp A show early post-infection defects (Thali et al, 1994). While mutations in the C-terminal region of CA disrupt Gag-Gag interactions and thus efficient release of the progeny particles, mutations in the N-terminal region cause no such decrease in the efficiency of release of HIV virions (Freed, 1998). By contrast, these mutations are known to disrupt the normal core condensation process thus interfering with the viral maturation and subsequent infectivity (von Schwedler

et al, 1998). Thus the N-terminal region of CA is essential for the proper functioning of the viral machinery at early times post-infection.

NC or p7 is the third cleavage product of Pr55^{GAG}. NC contains two zinc-finger motifs within its sequence. These motifs are essential for the specific binding and thus incorporation of the viral RNA into the budding virion (Aldovini & Young, 1990; Schwartz et al, 1997; Zhang & Barklis, 1997). NC has also been shown to have a role in the process of RNA dimerisation (Darlix et al, 1990; Derocquigny et al, 1992) and thus thermodynamic stabilisation of the RNA structure through a process called RNA maturation (Feng et al, 1996b). The N terminal 'I' (Interaction) domain of NC has been implicated as playing a role in efficient virus assembly and release (Bennett et al, 1993; Jowett et al, 1993). In addition to its role as a part of the Gag precursor, the mature NC protein is also involved in important steps in the viral replication cycle. It is essential for the binding of tRNA^{Lys3}, the primer for HIV-1 reverse transcription, to the primer binding site located near the 5' end of the HIV genome (Barat et al, 1993; Derocquigny et al, 1992). Thus NC plays a role in the initiation of reverse transcription and efficient strand transfer (Allain et al, 1994) during the reverse transcription process.

The final product of Pr55^{GAG} cleavage is p6. It is located at the C-terminal end of the Pr55^{GAG} sequence. Mutations in p6 have been shown to hamper efficient release of HIV virions pointing to a role for p6 in the late budding stage which is discussed in detail in section 1.11.5 (Gottlinger et al, 1991). The viral accessory protein Vpr has been shown to get incorporated into HIV virions through a specific interaction with the p6 domain of Pr55^{GAG} (Kondo et al, 1995), with the C-terminal domain of p6 being implicated in this function (Paxton et al, 1993). Thus Gag plays a very important role in the replication cycle of the virus.

1.10.1.2 Pol

The pol gene products are translated following a low-frequency ribosomal frame-shifting event that results in the formation of a 160 kDa precursor Pr160^{GAG-POL}, which contains the viral enzymes protease (PR), reverse transcriptase (RT) and integrase (IN) in addition to Pr55^{GAG} (Fig. 1.4) (Jacks et al, 1988; Wilson et al, 1988). The ratio of synthesis of Pr55^{GAG} to Pr160^{GAG-POL} is approximately 20:1 (Jacks et al, 1988; Wilson et al, 1988).

Cleavage of the Gag-Pol precursor into component Pol proteins is brought about by the aspartic protease PR (Peng et al, 1989). Dimerised PR first cleaves itself out of the precursor and subsequently fragments the Pr160^{GAG-POL} in an ordered process which ultimately results in the formation and release of fully infectious HIV particles (Graves et al, 1988; Mous et al, 1988). Inhibition of PR has no effect on the formation and release of virus particles but the process of viral maturation is affected resulting in the production of non-infectious virions. For the HIV replication cycle to be successful, it is critical that the stoichiometry of component Gag and Gag-Pol proteins is correct (Felsenstein & Goff, 1988). Hence the rate at which PR cleaves these protein precursors into mature proteins is very important. A small change in the amount of PR causes a huge difference in the quantity of the final products of cleavage ultimately affecting viral infectivity and morphology (Frankel & Young, 1998).

The second pol product is reverse transcriptase (RT). It is a heterodimer which has RNA and DNA dependent DNA polymerase as well as RNase H enzyme activities (Goff, 1990; Sarafianos et al, 2009). The heterodimer consists of two asymmetric but related subunits p66 and p51. The p51 subunit is cleaved from p66 by the viral protease after the formation of a p66/p66 homodimer (Sluis-Cremer et al, 2004). The

p66 subunit is 560 residues long, consists of two domains, polymerase and RNase H and acquires a right hand conformation when in solution (Sarafianos et al, 2009). The polymerase domain is composed of four sub-domains consisting of fingers (residues 1-85 and 118-155), palm (residues 86-117 and 156-236), thumb (residues 237-318) and the RNaseH connection (residues 319-426) (Jacobomolina et al, 1993; Kohlstaedt et al, 1992). All the enzymatic activities of RT are exhibited by the p66 subunit. In contrast, the p51 subunit plays a more structural role. It is 440 residues long and stabilizes the heterodimer (Herschhorn & Hizi, 2010). Interestingly the p51 and p66 share the same four domains (fingers, palm, thumb and connection) and also fold in a similar way but the relative position of the domains in the two subunits is different resulting in a different tertiary structure (Herschhorn & Hizi, 2010). The nucleic acid binding cleft of RT is composed primarily of the p66 fingers, palm, thumb, connection and RNase H domains. However the base of the binding cleft is made up of the connection and thumb domains of p51 (Sarafianos et al, 2009). Nucleic acids bind RT in such a way that they are simultaneously in contact with both the polymerase and RNase H active sites. Upon binding of a nucleic acid the p66 thumb changes its conformation from 'closed' where it is in contact with the fingers to 'open' where it moves 30° away from them (Hsiou et al, 1996; Rodgers et al, 1995). This initiates the process of reverse transcription that is discussed in detail in section 1.11.2. The end result of the reverse transcription process is the formation of a double-stranded cDNA copy of the HIV genome from single-stranded positive sense RNA (Gilboa et al, 1979). RNase H is essential for the cleavage of the RNA associated with the first DNA strand synthesised thereby allowing the single stranded DNA to synthesise its complementary strand (Hansen et al, 1988). The first DNA strand is primed by the RNA primer tRNA^{Lys3} and the second strand by the

polypurine tract which is resistant to digestion by RNase H (Pullen & Champoux, 1990; Schultz & Champoux, 2008). As RT has no proof reading activity, reverse transcription is an error prone process resulting in the introduction of a number of changes into every new copy of the genome synthesised (Bebenek et al, 1989).

The third and final product of pol is integrase (IN). IN integrates proviral DNA into the genomic DNA of the host cell. IN possesses two major catalytic activities namely 3' processing involving endonucleolytic cleavage at 3'-hydroxyl ends of the viral genome and strand transfer involving integration of the processed viral DNA into host DNA by trans-esterification (Delelis et al, 2008). The protein has 3 domains: 1) The N-terminal domain contains the zinc-binding site involved in multimerisation which is essential for IN function (Zheng et al, 1996) (2) the central catalytic domain which is critical for IN activity and 3) The C-terminal DNA binding domain which is significant for the stability of pre-integration complexes (PIC) (Delelis et al, 2008).

1.10.1.3 Env

The env gene encodes the glycoprotein precursor gp160, which is cleaved by the host protease enzyme furin into two proteins SU/gp120 and TM /gp41 (Fig. 1.4) (Freed & Martin, 1995a).

Glycoprotein precursor gp160 is synthesised and co-translationally glycosylated on the rough endoplasmic reticulum (ER) and is transferred into the lumen of the ER. The stop transfer sequence is located in the central portion of the transmembrane domain of gp160. The glycosylation of the gp160 monomers by addition of high mannose sugars shortly after synthesis is followed by the oligomerisation of the individual precursor molecules. Oligomerisation is said to be essential in the transfer of the precursor from the ER to the golgi complex (Willey et al, 1991). Once in the

golgi complex, some of the ER acquired high mannose oligosaccharide side chains are converted to more complex hybrid/terminally glycosylated forms and gp160 is proteolytically cleaved into gp120 and gp41 (Freed & Martin, 1995a; Willey et al, 1988). The Env glycoprotein is extensively glycosylated with 24 glycosylation sites present in gp120 and 4 sites within the gp41 sequence. Carbohydrates amount to almost 45-50% of the final molecular weight of the fully formed Envelope glycoprotein (Allan et al, 1985). The cleavage of the Env glycoprotein precursor at the golgi complex is inefficient and is catalysed by a host protease at a Lys/Arg-X-Lys/Arg-Arg motif that is highly conserved in viral Env glycoprotein precursors (McCune et al, 1988). The cleavage is said to be catalysed by furin or a furin-like cellular protease (Hallenberger et al, 1992). Following cleavage into gp120 and gp41, the non-covalently associated oligomeric proteins are transported to the plasma membrane where they get incorporated into the budding progeny virions.

These proteins remain associated with the lipid bilayer of cells infected with HIV as well as projecting from the virion surface as spikes. SU lies on the outer surface of the lipid bilayer and is important in CD4 receptor recognition, cell attachment and entry (Klatzmann et al, 1984; Sattentau & Moore, 1991). TM is embedded within the lipid bilayer and is involved in virus-host cell membrane fusion and entry (Gallagher et al, 1989; Kowalski et al, 1987).

Env plays a major role in the viral entry process (section 1.11.1) and getting an insight into the domain organisation of Env is critical to understanding the process of viral entry. The gp120 unit consists of five conserved (C1-C5) and five variable (V1-V5) domains (Starcich et al, 1986). The conserved regions contain many domains which are critical for binding to host cells. In contrast, the variable regions (V1-V4) form exposed loops and play a role in countering the humoral immune response by

constantly changing epitopes. In addition, V1/V2 and V3 play a very important role in gp120 binding to the co-receptor (Tilton & Doms, 2010). The gp41 unit consists of a large extracellular ectodomain, a transmembrane domain spanning the viral membrane and a cytoplasmic domain on the inside of viral membrane. The ectodomain consists of an N-terminal fusion peptide and two heptad repeat regions (HR1 and HR2) which are critical for the fusion process (Dubay et al, 1992; Tilton & Doms, 2010).

The growing genetic divergence between different HIV isolates can be attributed primarily to sequence variation in env (Lynch et al, 2009). Env is a major target for the humoral immune response against HIV and many neutralising antibodies are generated in humans against gp120. However the virus evades the antibody response by altering the gp120 sequence to generate antibody escape mutants (Frost et al, 2005).

1.10.2 Regulatory genes

In addition to Gag, Pol and Env which are the proteins expressed by all retroviruses, complex retroviruses like HIV also encode other regulatory and auxiliary proteins which perform diverse functions *in vivo*.

1.10.2.1 Tat (Transcriptional activator)

HIV-1 Tat acts as a transactivator of various viral and cellular genes (Ju et al, 2009; Mahlknecht et al, 2008). The tat mRNA is modified post-transcriptionally by a double splicing event (Fig. 1.4) (Pugliese et al, 2005; Romani et al, 2010). Two important functional domains of Tat have been identified, the first is the N-terminal cysteine rich activation domain involved in interactions with various cellular factors and the second is the arginine-rich RNA binding domain involved in nuclear

localisation and binding to the transactivation response element (TAR) of the LTR (Rana & Jeang, 1999; Romani et al, 2010). Tat is known to influence mRNA transcription and elongation. Tat recruits cellular proteins to relieve the repression of viral LTR so that the viral promoter can induce expression of the viral genes (Richman et al, 2009). It binds to the RNA stem loop structure TAR located at the 5' end of all initiated viral RNA transcripts, recruiting the cellular cyclin T1 and CDK9 proteins in the process and resulting in the phosphorylation of the C-terminal domain of RNA polymerase II which potentiates the enzyme to synthesise full-length HIV transcripts (Williams et al, 2006; Zhou et al, 2003).

1.10.2.2 Rev (Regulator of viral expression)

Rev is known to regulate the switch between early expression of the Tat, Rev and Nef proteins of HIV and the later Rev-dependent expression of Gag, Pol, Env, Vif, Vpr, Vpu and the production of viral genomic RNA for packaging into newly formed virions (Fig. 1.4) (Cullen, 1992; Emini, 2002). This switch is accomplished by allowing the nuclear export of partially spliced and unspliced viral mRNAs (Malim et al, 1989). Thus early viral transcripts produced in the absence of Rev are spliced resulting in the expression of Tat, Rev and Nef (Fig. 1.4). As the amount of Rev increases it prevents the splicing and activates the export of unspliced viral mRNAs and late expression of viral structural proteins (Fig. 1.4). Rev interacts with the Rev response element (RRE) located in the env sequence to promote the stability of the unspliced mRNAs, their export from the nucleus and their translation into proteins (Malim et al, 1989).

1.10.3 Accessory genes

In addition to the various structural and regulatory genes discussed above, the HIV genome also contains four other genes which although they are dispensable for growth in tissue culture systems, have been shown to be critical for the establishment of a successful infection *in vivo* (Trono, 1995). The genes *vif*, *vpr*, *nef* and *vpu* are collectively called the accessory genes of HIV. Of these four only Nef has Rev-independent expression early in the regulatory phase of infection. Vif, Vpr and Vpu are expressed late in a Rev-dependent fashion during the structural protein phase of viral gene expression (Fig. 1.4).

1.10.3.1 Nef

Nef is an N-terminally myristoylated accessory protein of HIV-1 produced at all stages of viral replication and packaged non-specifically into HIV virions in low amounts (Li et al, 2005). It serves as an adapter protein and diverts host cellular proteins to perform functions that aid virus replication in host cells (Geyer et al, 2001). Nef is known to downregulate the cell surface expression of both CD4 (Garcia & Miller, 1991) and Class-1 MHC molecules (Schwartz et al, 1996). This is achieved by internalisation followed by lysosomal degradation of these molecules which in turn leads to inhibition of CTL-mediated lysis of HIV-1 infected cells and aids viral pathogenesis (Collins et al, 1998). Nef enhances viral infectivity by aiding `uncoating of the virion core and proviral DNA synthesis (Aiken & Trono, 1995; Bukovsky et al, 1997). Nef may also be responsible for cellular activation and in turn disease progression through cellular kinases Pak2 (Raney et al, 2005; Renkema et al, 1999) and Hck (Foster & Garcia, 2008; Ye et al, 2004). Nef also regulates cholesterol trafficking by binding to newly synthesised cholesterol and transporting it to the site of viral budding in HIV-infected cells (Li et al, 2005; Zheng et al, 2003).

Cholesterol has been shown to be important in the HIV life cycle as depletion of cellular cholesterol leads to a decrease in HIV particle production (Maziere et al, 1994). Thus Nef performs a number of important functions allowing viral replication to continue normally inside host cells.

1.10.3.2 Vpr

Vpr is a small basic nuclear protein expressed from a singly spliced mRNA (Fig. 1.4) (Emini, 2002; Li et al, 2005). It is expressed late in the viral replication cycle but is present in the early post-entry phase as it is incorporated into the budding progeny HIV virions through a direct interaction with the carboxy-terminal p6 domain of the Pr55^{GAG} precursor (Bachand et al, 1999; Le Rouzic & Benichou, 2005). Vpr is efficiently packaged into the progeny virions with a Vpr:Gag ratio of 1:7 (Muller et al, 2000). After entry into host cells, the viral capsid gets uncoated and the single stranded genomic RNA is reverse transcribed to produce double-stranded DNA. The viral cDNA then associates with many viral and host cellular proteins to form the viral pre-integration complex (PIC) (Le Rouzic & Benichou, 2005). Vpr is a part of the PIC and enhances the transport of viral DNA from the cytoplasm to the nucleus for subsequent integration in non-dividing cells (Heinzinger et al, 1994). Another important function of Vpr is that of inducing the arrest of infected proliferating T cells in the G2 phase of the cell cycle (Jowett et al, 1995). The HIV-1 LTR is more active in the G2 phase of the cell cycle and thus Vpr creates a favourable environment for the efficient transcription of viral genes by inducing cell cycle arrest (Goh et al, 1998).

1.10.3.3 Vpu

Vpu is produced late in the replication cycle and is translated from a vpu-env bicistronic mRNA (Fig. 1.4) (Nomaguchi et al, 2008; Schwartz et al, 1990). It is not

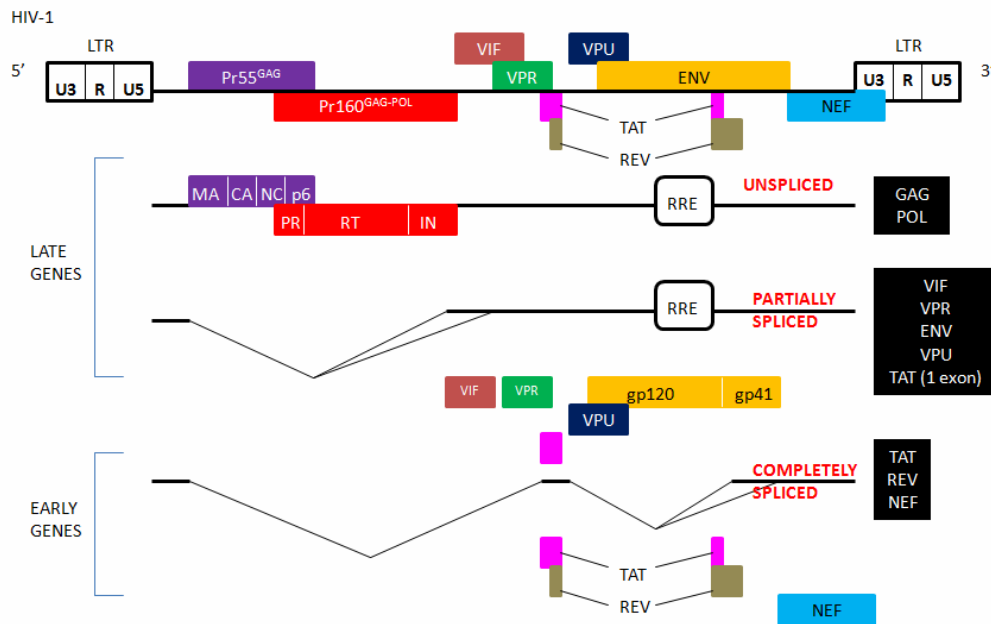


Figure 1.4 Gene organisation of HIV with the mechanism of expression of the early and late genes in the replication cycle: The HIV-1 integrated provirus encodes 9 genes in total, 3 of which the gag, pol and env are the basic genes present in all retroviruses. In addition, HIV encodes 2 regulatory genes; tat and rev and 4 accessory genes; vif, vpr, vpu and nef. Rev acts as a switch to modulate the expression of the various genes at the right time in the HIV replication cycle through its interaction with the Rev response element (RRE) located in the env sequence to promote the stability of the unspliced mRNAs, their export from the nucleus and their translation into proteins. The first viral RNAs to be transcribed in the absence of Rev are spliced and hence the first viral proteins to be expressed are Tat, Rev and Nef as they are synthesised from fully spliced mRNAs and are referred to as early gene products. The level of the regulatory protein Rev determines the splicing of the RNAs. Thus Rev is responsible for the transition from the early regulatory phase of HIV infection where it directs the splicing of the majority of viral RNAs to the late structural phase of infection where it directs the transcription of partially spliced as well as full-length mRNAs which are translated into the structural proteins of HIV responsible for virion assembly. The late gene products of HIV are Gag, Pol, Env, Vpr, Vif and Vpu.

packaged into virions but is found only in virus infected cells (Nomaguchi et al, 2008). Vpu is made up of two distinct domains, an N-terminal transmembrane domain and a cytoplasmic domain (Schubert et al, 1996). The cytoplasmic domain of Vpu, together with Nef and Env, is involved in the down-regulation of CD4 at the cell surface. While Nef enhances clathrin-mediated endocytosis of CD4 molecules at the cell surface and forwards them to the lysosomes for degradation and Env forms a complex with the newly synthesised CD4 in the ER, Vpu targets the CD4 in the Env-CD4 complex mediating its subsequent degradation in the ER (Garcia & Miller, 1991; Sattentau & Moore, 1991; Willey et al, 1992). This releases Env from the CD4 complex and allows its proper incorporation into virions. Vpu is indispensable for normal virion release from HeLa cells, monocyte-derived macrophages and primary T-cells (Vpu-dependent cells) (Klimkait et al, 1990). By contrast, Vpu is not required for efficient virion release from 293T, COS and Vero cells (Vpu-independent cells) (Nomaguchi et al, 2008). Thus the virion release function of Vpu is cell type dependent. It has been shown recently that Vpu is required to counteract the host-cellular virion release inhibitor Tetherin, with the N-terminal transmembrane domain being involved in this function. This is discussed in greater detail in section 1.12.3 (Neil et al, 2008).

1.10.3.4 Vif

The Virus Infectivity Factor (vif) is one of the accessory genes of HIV (Strebel et al, 1987). It codes for the Vif protein which is a 192 amino acid long 23kDa phosphorylated protein conserved among all primate and most other lentiviruses. Vif is expressed at late stages of the viral replication cycle and is found in both the nucleus and cytoplasm of the infected host cell (Goncalves et al, 1994). It co-localises with the Gag protein in the core of the HIV virion (Bardy et al, 2001). The

characteristic properties and functions of Vif are discussed in greater detail later in this chapter.

1.11 The replication cycle of HIV-1

The HIV replication cycle can be divided into the following stages (Emini, 2002) for simplistic understanding of the mechanisms involved (Fig. 1.5):

1.11.1 Virus entry and cell tropism

HIV-1 is primarily known to infect CD4⁺ T-cells and macrophages which are its host cells in humans. Infection of dendritic and langerhans cells of the macrophage lineage is important for the transmission of virus infection as these cells are involved in the presentation of antigen to T-cells (Knight et al, 1990; SotoRamirez et al, 1996). In addition, HIV also infects macrophages and microglial cells in the central nervous system (Johnson, 1995). The viral envelope surface glycoprotein gp120 is crucial for the attachment of virus to its receptors. The process of viral entry requires the presence of the CD4 cell surface receptor (Stein et al, 1987) and at least one of the G-protein coupled co-receptors (GPCRs) with spanning seven-transmembrane regions on the surface of host cells (Feng et al, 1996a). The GPCRs play a crucial role in viral tropism to certain cell types.

Most HIV strains which are able to infect cultured T-cell lines through an interaction of gp120 with the cellular receptor CD4 and co-receptor CXCR4 are called T-tropic or X4 viruses (Bleul et al, 1996; Feng et al, 1996a). These viruses also induce syncytia and hence are called ‘Syncytia Inducing (SI)’ viruses and are usually seen in late stage HIV disease when the immune system is waning (Connor et al, 1997;

Tersmette et al, 1988). On the other hand, some viruses use CCR5 as their chemokine co-receptor for entry and don't induce syncytia in T-cell lines (Deng et al, 1996; Dragic et al, 1996; Tersmette et al, 1988). These viruses are called Non-syncytia Inducing (NSI) or M-tropic (macrophage-tropic) or R5 viruses (Coakley et al, 2005). R5 HIV-1 is the characteristic strain found in the early asymptomatic stage of the infection; it lasts throughout the disease and is the predominant strain transmitted between individuals (Schuitemaker et al, 1992). Some isolates are able to bind with both CCR5 and CXCR4 with equal efficiency and hence are called R5X4 strains (Doranz et al, 1996). Although other chemokine receptors are also present on the surface of cells, all of the known strains of HIV-1 use CCR5 and/or CXCR4 as their co-receptors for attachment and entry (Choe et al, 1996; Doranz et al, 1996).

T-tropic viruses can infect both resting and activated T-cells. However there is a post-entry block to virus replication in resting T-cells and so replication does not occur in these cells (Bukrinsky et al, 1991; Stevenson et al, 1990). Similarly X4 viruses can infect macrophages but again virus replication cannot proceed normally due to a block at the post-entry level (Schmidtayerova et al, 1998). M-tropic viruses can infect macrophages and activated T-cells but not resting T-cells due to the low expression of CCR5 on the surface of these cells (Bukrinsky et al, 1991; Spina et al, 1995).

HIV entry into the cell is a multi-step process. The initial interaction between HIV and the target cell may be facilitated by positively charged domains on gp120 and negatively charged proteoglycans on the cell surface or by specific interactions with DC-SIGN on the dendritic cell surface (Geijtenbeek et al, 2000; Tilton & Doms, 2010). These proteins don't play a role in viral entry into the cell but do increase the efficiency of the process. The first specific stage of viral entry involves an interaction

between viral gp120 and the CD4 cellular receptor. The domain structure of Env has been discussed in detail in section 1.10.1.3. The epitopes within gp120 involved in this interaction are highly conserved and are discontinuous in the protein sequence (Kwong et al, 1998). The binding of gp120 to CD4 induces a dramatic conformational change in gp120 which enhances its binding affinity for the chemokine co-receptors (Wu et al, 1996). Later, gp120 interacts with either CXCR4 or CCR5. Thus the gp120-CD4 interaction is a pre-requisite for the formation and exposure of the co-receptor binding site. Binding of gp120 to the co-receptor results in further conformational change in Env which in turn leads to the exposure of the hydrophobic fusion peptide of gp41 and its insertion into the host cell plasma membrane (Tilton & Doms, 2010). Following insertion, the heptad regions HR1 and HR2 of gp41 undergo an energetically favourable structural rearrangement bringing the transmembrane region of gp41 which is embedded in the viral membrane into close proximity to the fusion peptide which is now inserted into the host cell membrane. This re-positioning of gp41 results in the formation of the fusion pore and entry of the capsid into the cell (Chan & Kim, 1998; Kwong et al, 1998; Stein et al, 1987; Tilton & Doms, 2010).

1.11.2 Reverse Transcription

Following entry of the viral core into the cytoplasm of the target cell, the process of reverse transcription (RTN) converts the ssRNA of the input viral genome to dsDNA (Fig. 1.5) (Gilboa et al, 1979). Reverse transcriptase (RT) is the enzyme which carries out the whole process after associating with the viral genomic RNA and a primer to initiate RTN. HIV-1 specifically uses host cell tRNA^{Lys3} as a primer for RTN (Kleiman et al, 1991). The 3' 18 nucleotides of this tRNA are complementary

to a site near the 5' end of the HIV-1 RNA genome known as the primer binding site enabling the hybridisation of the tRNA to this site (Ratner et al, 1985).

The first stage of RTN involves synthesis of the viral (-) DNA strand from the (+) strand RNA genome. The 5' end of the viral RNA genome consisting of the 5' unique sequence (U5) and the 5' repeat region (R) is synthesised by extending the 3'-OH end of the tRNA primer (Herschhorn & Hizi, 2010). As RT reaches the end of the genome, its RNase H activity hydrolyzes the genomic RNA strand from the newly synthesised RNA/DNA hybrid. This leaves the (-) sense DNA strand designated as (-) strand strong stop DNA (sssDNA) which has sequences which are complementary to the identical repeat (R) sequences located at both the 5' and 3' ends of the viral RNA genome. The (-) sssDNA then hybridizes to the 3' end R sequences of the viral genomic RNA, this process being designated first (-) strand transfer (Luo & Taylor, 1990; Peliska & Benkovic, 1992). After the DNA hybridizes to the 3' end R sequence of the genomic RNA, the (-) strand DNA synthesis proceeds and the copied RNA is degraded simultaneously by the RNase H activity of the RT (Sarafianos et al, 2009).

Some purine-rich sequences show high resistance to degradation by RNase H. One such sequence called the Major Polypurine Tract (PPT) is located at the 3' end of the viral genome and serves as the primer for the synthesis of second (+) strand DNA (Rausch & Le Grice, 2004). All retroviruses use the 3'PPT as the primer for (+) strand DNA synthesis but some retroviruses such as HIV-1 also have additional PPTs, the most notable of which is the central PPT (cPPT) located in the centre of the genome in the IN coding sequence (Charneau et al, 1992). HIV-1 uses both of these PPTs as primers for the initiation of second (+) strand synthesis.

(+) strand DNA synthesis begins at the PPT and proceeds by copying the (-) DNA strand to its 5' end. RNase H removes the primer tRNA from the RNA/DNA duplex and this facilitates second strand transfer where the partially synthesised (+) DNA strand anneals with the (-) DNA strand at the complementary primer binding site segments (Peliska & Benkovic, 1992). The (+) and (-) DNA strand synthesis is then completed by each strand serving as a template for the other. The end result is the generation of dsDNA containing an LTR at either end.

1.11.3 Nuclear import of proviral DNA and integration

Once the dsDNA is synthesised, it gets incorporated into a complex with other viral proteins namely RT, IN, NC, MA and Vpr (Bukrinsky et al, 1993b; Heinzinger et al, 1994). This complex is called the pre-integration complex (PIC). For the virus replication to start, the newly formed PIC should be transported to the nucleus (Fig. 1.5). This is achieved by distinct nuclear localisation signals (NLS) on both MA and Vpr whose function though synergistic, is independent of each other (Bukrinsky et al, 1993a; Heinzinger et al, 1994). The PIC can be imported through the nuclear pore into the nucleus without the destruction of the nuclear membrane, which helps HIV-1 to replicate in even terminally differentiated and static cells, a feature that favours efficient viral replication and spread (Emini, 2002).

Following transport of the PIC to the nucleus, IN undergoes a series of steps to catalyse integration of the proviral DNA into the host chromosome. The first step involves endonucleolytic cleavage of a dinucleotide from the 3' end of each strand leaving the viral DNA with 3' CA_{OH} overhangs referred to as 3' processing (Delelis et al, 2008; Katz & Skalka, 1994). The next step designated DNA strand transfer involves cleavage of the host chromosome by nucleophilic attack from the 3'

hydroxyl overhangs of the viral DNA and subsequent ligation of the viral DNA to the 5' end of the target DNA at the cleavage site. The gaps generated in the target DNA as a result of the integration are repaired by host enzymes (Frankel & Young, 1998; Miller et al, 1995). The end result is flanking of the integrated proviral DNA with 5 base pair repeats of the target site DNA (Delelis et al, 2008).

1.11.4 Viral gene expression

Following integration of proviral DNA into the host cell genome, HIV-1 uses the cellular machinery for its replication. Host RNA Polymerase II is responsible for transcribing the DNA into mRNAs which are either translated into viral proteins or are incorporated into the progeny virions as viral genomic RNA (Emeni, 2002). The rate of transcription depends on cellular transcription factors such as NF- κ B, NFAT-1 and AP-1, particularly in the early transcription phase prior to the expression of Tat (Kingsman & Kingsman, 1996). Cellular activation signals result in these transcription factors binding the LTR and initiating viral RNA transcription (Cullen, 1991). After the expression of Tat, the efficiency of viral transcription increases (Laspia et al, 1989). For the whole transcription process to proceed smoothly there needs to be a dynamic collaboration and balance between the host transcription factors mentioned above and the viral regulatory proteins Tat and Rev. The first viral mRNAs to be transcribed are spliced; this results in the first HIV proteins to be synthesised being Tat, Rev and Nef since they are expressed from fully spliced mRNAs and are referred to as early gene products of HIV. The level of the regulatory protein Rev determines the splicing of the RNAs (Malim et al, 1989). Thus Rev is responsible for the transition from the early regulatory phase of HIV infection where it directs the splicing of the majority of viral RNAs to the late

structural phase of infection where it directs the transcription of full-length mRNAs which are translated into the structural proteins of HIV responsible for virion assembly (Cullen, 1992).

1.11.5 Virion assembly and release

Pr55^{GAG} is responsible for mediating the assembly and release of progeny HIV-1 from host cells (Gheysen et al, 1989). The precise sequence of events leading to HIV assembly has not been determined and many events are said to occur simultaneously within the infected cell. Different regions of Pr55^{GAG} perform different critical functions during particle assembly. MA is responsible for Gag targeting, membrane binding and Env incorporation (Bryant & Ratner, 1990; Dorfman et al, 1994b). The C-terminal region of CA along with the N-terminal I domain of NC is believed to mediate Gag multimerisation (Jowett et al, 1993; Zhang et al, 1996). The specific incorporation of viral genomic RNA is mediated by the zinc finger domains of NC (Schwartz et al, 1997), while p6 is responsible for the recruitment of the cellular protein complexes critical for efficient particle release (Gottlinger et al, 1991; Ono, 2010).

Initial Gag-Gag interactions are believed to occur in the cytoplasm while the formation of higher ordered structures appears to take place at the plasma membrane (Ono et al, 2000). Gag localises to the membrane micro-domains from where budding of progeny virions takes place (Holm et al, 2003). The Gag/Gag interactions also stimulate the formation of complexes between Gag-Pol and Gag (Bukrinskaya, 2004). The Gag/Gag-Pol complexes are directed to the plasma membrane by the membranotropic signal present on the MA region of Pr55^{GAG} (Halwani et al, 2003). The Gag/Gag-Pol ratio is essential for RNA dimerisation, a process that is required

for efficient RNA packaging and the integrity of the newly formed virions (Sakuragi et al, 2003; Shehu-Xhilaga et al, 2001).

The HIV accessory proteins Vpr, Vif and Nef are also packaged into HIV virions. Vpr is packaged into the virions through an interaction with the p6 domain of Pr55^{GAG} in a ratio of 1:7 (Bachand et al, 1999; Muller et al, 2000). It has an important role in the post-entry stage of infection which has been discussed in section 1.11.3. Vif also gets incorporated into the progeny virions through an interaction with Pr55^{GAG} (Bardy et al, 2001; Bouyac et al, 1997a). Vif is believed to be important in virion core stability and infectivity (Bukrinskaya, 2004). Virions produced in its absence have reduced infectivity. Nef is packaged into progeny virions non-specifically and is believed to play a role during the virion uncoating process discussed in detail in section 1.10.3.1 (Aiken & Trono, 1995; Li et al, 2005).

In addition to viral proteins, some cellular entities are also packaged into virions. One of them, Cyp A is packaged through an interaction with the CA domain of Pr55^{GAG} and promotes viral infectivity (discussed in section 1.10.1.1) (Thali et al, 1994). In addition the cellular RNase L inhibitor HP68 is associated with pre-assembled viral complexes of Gag, Gag-Pol and Vif and is believed to play a role in the formation of immature virus particles (Zimmerman et al, 2002). Numerous cellular RNAs also get incorporated into progeny virions, one of them being tRNA^{Lys3}, which is the primer that initiates reverse transcription in the target cell post-entry. It gets incorporated through an interaction with specific RT sequences within Gag-Pol (Mak et al, 1994).

Budding of progeny virions is initiated following the association of genomic RNA/polyprotein complexes with the Env glycoproteins at the plasma membrane

(Fig. 1.5) (Emini, 2002). Two domains have been identified in the p6 sequence, PTAP and LYPX_nL, which mediate the efficient release of virions (Demirov et al, 2002; Strack et al, 2003). Similar conserved domains like PPPY and YPDL have also been identified in other viruses and are collectively referred to as 'late' domains essential for the late budding stage of the virus (Bieniasz, 2006). The current view in the literature is that the late domains of HIV hijack the cellular machinery responsible for the inward vesiculation in late endosomes which generates multivesicular bodies (MVBs) and use this machinery for viral release from the cell surface (Fujii et al, 2007).

The class E Vacuolar protein sorting (Vps) proteins in mammals are required for the formation of three high molecular weight endosomal sorting complexes required for transport (ESCRT-1, 2 and 3) of cargo like activated receptors to the MVBs before their degradation in lysosomes (Fujii et al, 2007). The cycling of ESCRT from the limiting membrane of MVB to the cytoplasm is catalysed by the ATPase Vps4. Several studies have shown an interaction of the PTAP domain of p6 with Tsg101 (tumor susceptibility gene101), a component of the ESCRT-1 complex and demonstrated the importance of this p6-Tsg101 interaction in HIV budding and release (Demirov et al, 2002; Garrus et al, 2001; VerPlank et al, 2001). The recruitment of charged multivesicular body proteins (CHMPs), a component of the ESCRT-3 complex (Martin-Serrano et al, 2003; von Schwedler et al, 2003) and the activity of ATPase Vps4 are also required for this process (Kieffer et al, 2008). On the other hand, the LYPX_nL motif of p6 has been demonstrated to bind with Alix, a protein playing a role in endosomal metabolism (Zhai et al, 2008). The p6-Tsg101 interaction is thought to play a primary role while the LYPX_nL-Alix

interaction has been shown to have a supporting role in virus release and budding from the cell surface (Dussupt et al, 2009).

Prior to the process of budding, the Gag-Gag and Gag-Pol complexes are selectively associated with membrane micro-domains predominantly made up of cholesterol, glycosphingolipids and sphingomyelin called lipid rafts (Aloia et al, 1993; Holm et al, 2003). It is believed that these lipid rafts act as scaffolds for the assembly and subsequent budding of the immature virions from the cellular surface.

1.11.6 Maturation

The virions released from cells are immature and made up of precursors of structural proteins arranged in a spherical configuration in close association with the envelope. These spherical immature virions are processed and matured in a process called maturation (Fig. 1.5). Maturation is not essential for assembly or release but is critical for the infectivity of the newly released virions (Peng et al, 1989). During the process, large-scale reorganisation takes place in the structure of the virus particle by ordered cleavage of poly-protein precursors by viral enzyme protease (PR) (Kohl et al, 1988; Wiegers et al, 1998).

This cleavage of the Gag and Gag-Pol precursors takes place either at the plasma membrane immediately prior to budding or shortly after release of the virus particles (Kaplan et al, 1994). The first cleavages to occur are carried out by immature PR which is associated within the Gag-Pol precursor (Bukrinskaya, 2004). Two Gag-Pol precursors dimerise and are later cleaved to form the mature PR which is a dimer and is responsible for all subsequent cleavages (Tessmer & Krausslich, 1998). Ordered and accurate cleavage of the precursors is of prime importance for the infectivity of the mature virions. After activation of the mature PR, the first point of cleavage is

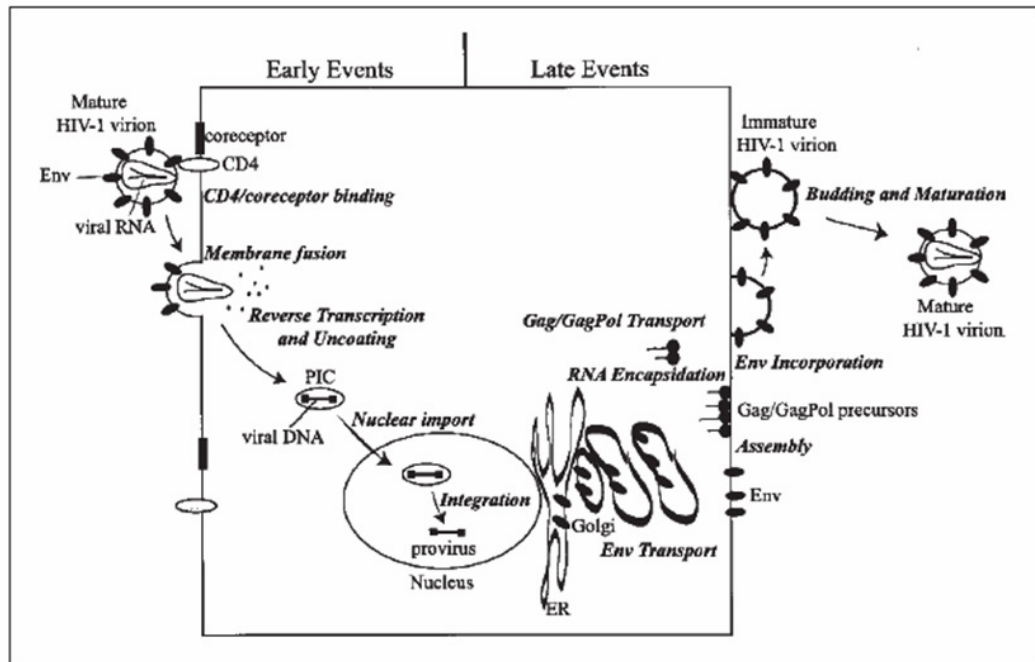


Figure 1.5 Schematic representation of the HIV-1 replication cycle: The major events occurring during the early and late stages of HIV-1 life cycle have been depicted above. (Figure adapted from: Freed EO, 2001)

between p2 and NC, the second between MA and CA and the last between CA and p2. The p2-NC cleavage in Gag is important for NC condensation to form the ribonucleoprotein (RNP) core (Wieggers et al, 1998). The last cleavage between CA and p2 triggers the formation of the conical CA shell over the NC/RNA core. This core condensation process is important for the conical, electron dense core morphology and hence infectivity (Wieggers et al, 1998).

The reorganisation and morphological changes in Gag also results in a change in the conformation of the packaged viral genomic RNA. The genomic RNA dimer is initially encapsidated in an extended conformation while later it adopts a condensed structure (Fu & Rein, 1993; Vogt, 1996). PR based cleavage is believed to be essential in this step as it is the mature NC that mediates this reorganisation of the genomic RNA (Darlix et al, 1995). It is this condensed RNA together with the associated NC which provides a scaffold for the ordered maturation of the virus particles making them infectious (Sheng et al, 1997).

1.12 Host cellular restriction factors affecting HIV infection

To date, there are three major known types of host cellular restriction factors that are known to counteract HIV infection at three different stages of the HIV life cycle mentioned above. Although it is believed that there is still a vast amount of information to be accessed and there could be many more restriction factors inherently present in humans that are yet to be elucidated. While these factors are constitutively expressed in some cell types, they can be activated in other cells by type I interferon treatment.

1.12.1 APOBEC3 family of cytidine deaminases

The APOBEC3 family of cytidine deaminases, their antiviral activities and the role of viral accessory protein Vif in overcoming the inhibition exerted by them is discussed in detail in sections 1.14 and 1.15.

1.12.2 Trim5 α

The role played by Trim5 α proteins in retroviral restriction was first evident when it was found out that these proteins were responsible for the inability of HIV-1 to establish a successful infection in old world monkeys (Stremlau et al, 2004). Similarly human and primate cells were found to be refractory to infection by certain strains of Murine Leukemia Virus (MLV) or Equine Infectious Anemia Virus (EIAV) (Hatzioannou et al, 2004; Keckesova et al, 2004; Perron et al, 2004; Yap et al, 2004).

The molecular details of the blockade to viral replication exerted by Trim5 α is not yet completely clear however a broad understanding is available. The C-terminal domain of Trim5 α directly recognises the incoming viral capsid causing an irreversible, lethal lesion within the capsids leading to their premature disassembly and proteasome-mediated degradation (Stremlau et al, 2006). This domain also governs the specificity of Trim proteins towards capsid proteins from certain viruses (Perez-Caballero et al, 2005). The Trim proteins also consist of a central coiled-coil domain responsible for multimerisation which is known to be essential for Trim activity (Neil & Bieniasz, 2009). In addition, they also contain an N-terminal 'effector' domain which increases the potency of inhibition mediated by Trim5 α (Perez-Caballero et al, 2005).

HIV has evolved to escape this restriction exerted by human Trim5 α by at least two different mechanisms. Certain mutations in the HIV capsid sequence prevent its recognition by human Trim proteins and thus prevent the subsequent premature capsid disassembly and degradation (Ylinen et al, 2005). The HIV capsid proteins are also known to bind Cyclophilin A, a host cellular protein, which coats the capsid and prevents its degradation by Trim5 α (Towers et al, 2003).

However, still a lot remains to be known about the exact mechanism of Trim5 α mediated post-entry inhibition of HIV replication, but it is one of the promising targets for future antiretroviral drug development.

1.12.3 Tetherin

Tetherin is a recently discovered antiretroviral restriction factor that acts at the virion release stage of the HIV life cycle (Neil et al, 2008; Van Damme et al, 2008). Tetherin crosslinks the mature virus particles to the cell membrane and thus prevents their release (Neil et al, 2006). The tethered virus particles are then reinternalised and directed to the late endosomes where they are either retained or destroyed by the lysosomal enzymes (Neil et al, 2006).

However HIV has also evolved an escape mechanism from this tetherin-mediated restriction. HIV encodes the viral accessory protein Vpu which specifically sequesters tetherin from the sites of viral assembly thereby preventing the adverse effects of tetherin on viral particle release (Neil et al, 2008). Vpu reduces the level of tetherin at the cell surface and forwards it to the 26s proteasome for degradation allowing productive viral replication to continue within the PBMCs (Goffinet et al, 2009; Van Damme et al, 2008).

Thus HIV-1 Vpu is critical for counteracting the host cell restriction factor tetherin and the proper release of the viral particles formed at the cellular membrane.

1.13 The Virus Infectivity Factor of HIV

1.13.1 Introduction

The vif gene of HIV-1 was initially recognised as a short open reading (sor) in the centre of the HIV-1 genome, overlapping pol at its 5' end and vpr at its 3' end, which encodes a 192 amino acid protein (~23 Kdaltons) designated Vif (Kan et al, 1986; Lee et al, 1986; Ratner et al, 1985; Sanchez-Pescador et al, 1985; Wain-Hobson et al, 1985).

1.13.2 Cell type dependent requirement of HIV-1 Vif

Vif, which has homologues in all lentiviruses with the exception of equine infectious anaemia virus (EIV), is an abbreviation of virus infectivity factor and this stems from the fact that viral genomes with deletions of vif yield significantly lower levels ($>3\log_{10}$) of infectious virus when grown in either a range of so-called 'non-permissive' T-cell lines (eg: CEM, HUT78 and H9) (Fisher et al, 1987; Sodroski et al, 1986; Strebel et al, 1987) or crucially in peripheral blood mono-nuclear cells (PBMCs) (Gabuzda et al, 1994), the natural host cells for HIV-1 (Fig. 1.6). By contrast when such vif deleted virus is grown in other T-cell lines (eg: C8166, SupT1 and Jurkat) termed 'permissive' cells, the yield of virus is similar to that seen with wild type virus (Fan & Peden, 1992; Gabuzda et al, 1992) (Fig. 1.6). This dichotomy of behaviour in different host cells could be explained either by the synthesis of a Vif substitute in 'permissive' cells or alternatively the synthesis of an inhibitor of virus

replication by ‘non-permissive’ cells that Vif is required to overcome. Using heterokaryons formed between permissive and non-permissive cells the latter phenotype was found to be dominant and consequently that Vif is required by HIV-1 to overcome an inhibitor of viral replication expressed in PBMCs and non-permissive T-cell lines (Madani & Kabat, 1998; Simon et al, 1998). In an elegant study using subtraction cloning, Malim’s group identified the cellular inhibitor as CEM15 (Sheehy et al, 2002) (subsequently called APOBEC3G abbreviated to A3G) which was rapidly shown to be a member of the APOBEC3 family of cytidine deaminases and is discussed in greater detail in the later sections of this thesis.

1.13.3 General characteristics of the HIV-1 Vif protein

Vif is a basic phosphorylated protein expressed in the late stages of the virus replication cycle (Yang & Gabuzda, 1999; Zhang et al, 2000). The majority of Vif protein appears to localise to the cytoplasm and cellular membrane mainly co-localising with Pr55^{GAG} (Goncalves et al, 1994; Karczewski & Strebel, 1996; Simon et al, 1997).

Because of its highly insoluble nature and its tendency to aggregate, purification of the Vif protein has not been successful to-date and hence little structural data is available for this protein (Barraud et al, 2008). It shows no significant sequence similarity or regions of homology to any other known protein. It has therefore been very difficult to predict the various functional domains of Vif (Lake et al, 2003). However, biochemical analyses have revealed residues throughout the protein which are essential for a variety of Vif functions and interactions (Simon et al, 1999).

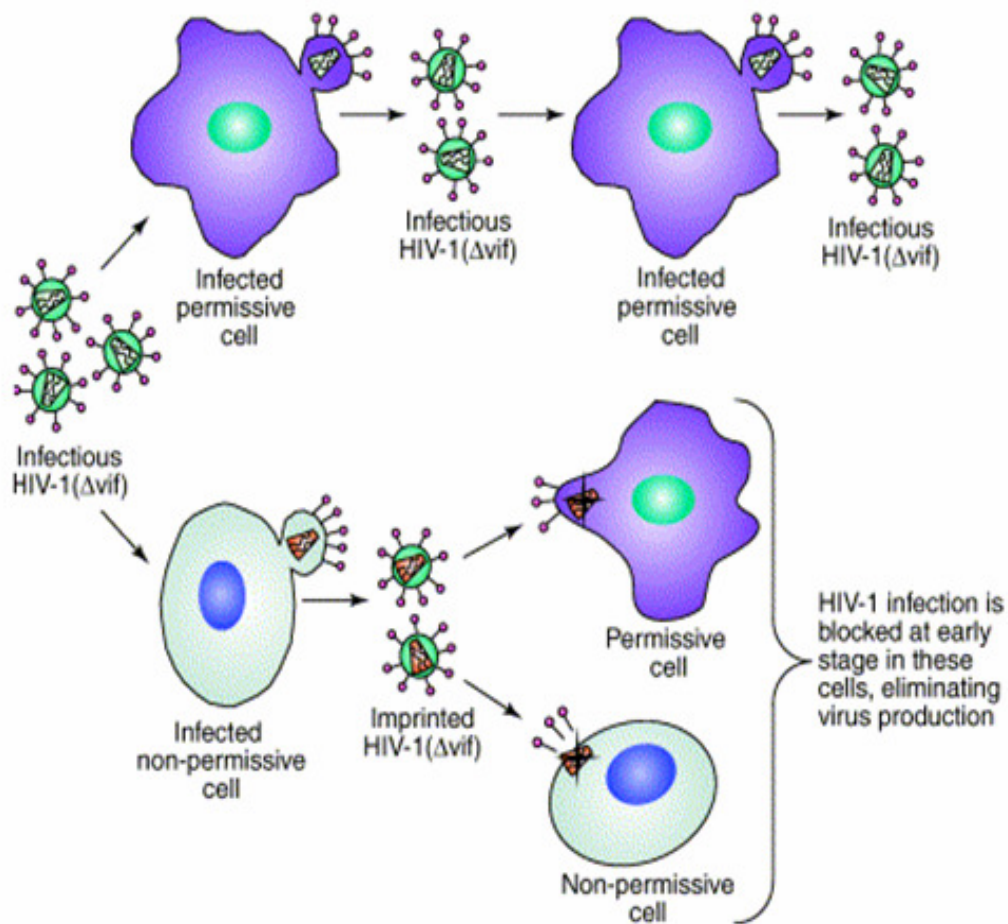


Figure 1.6 Cell type dependent requirement of HIV-1 Vif: When vif-deleted HIV-1 infects certain T-cell lines e.g. C8166, its growth, replication and infectivity is similar to wild type HIV-1, consequently these cells are referred to as 'Permissive' cells for the virus. By contrast, when vif-deleted HIV-1 is allowed to infect other T-cell lines eg H9 or more significantly, peripheral blood lymphocytes, the natural host cells for HIV-1 in humans, the first cycle of replication in these cells is normal. However, the resultant progeny virions are non-infectious; consequently these cells are called 'Non-permissive' cells for the virus. This difference in infectivity is due to the presence of an inhibitor of virus replication in the Non-permissive cells identified as belonging to the APOBEC3 family of proteins (Figure adapted from: Rose *et al*, 2004).

1.13.4 Conserved domains within the HIV-1 Vif protein

The Vif proteins of closely related HIV-1 strains are highly conserved while those from distantly related lentiviruses have diverged significantly (Barraud et al, 2008; Oberste & Gonda, 1992). Sequence alignment of HIV-1, HIV-2 and SIV shows certain conserved domains such as the N-terminal tryptophan-rich stretch, a HCCH motif and a SOCS-box motif (Barraud et al, 2008).

1.13.4.1 The N-terminal Tryptophan-rich region

The N-terminal tryptophan-rich region (residues 1 to 21) in the Vif sequence has been shown to play a role in Vif recognition and subsequent suppression of the host cell restriction factors APOBEC3G (A3G) and APOBEC3F (A3F) (Fig. 1.7) (Tian et al, 2006).

1.13.4.2 The HCCH motif

The HCCH motif (residues 108–139) consists of two conserved His/Cys pairs, the sequence and spacing of which is critical for Vif function (Fig. 1.7). Each of the residues within these two pairs was found to be important for the binding of Cullin 5 to Vif and the subsequent proteasomally mediated degradation of A3 proteins by the E3-ubiquitin ligase complex (Luo et al, 2005; Mehle et al, 2004a; Yu et al, 2004).

1.13.4.3 The SOCS-box motif

The highly conserved ¹⁴⁴SLQYLA¹⁴⁹ motif also called the SOCS-box is responsible for the binding of Vif to ElonginC, which further targets A3 antiviral factors to the proteasome for degradation (Fig. 1.7) (Mehle et al, 2004a; Mehle et al, 2004b; Yu et al, 2003; Yu et al, 2004).

1.13.5 Vif multimerisation

HIV-1 Vif protein has a tendency towards multimerisation which is reported to be crucial for viral infectivity (Yang et al, 2001). It has also been found that blocking Vif dimerisation prevents the Vif-mediated degradation of A3 proteins and in turn leads to the incorporation of these proteins in progeny HIV virions (Miller et al, 2007; Yang et al, 2003). Thus multimerisation of Vif is essential for the productive replication of HIV-1 inside host cells. The C-terminal domain of Vif, specifically the conserved ¹⁶¹PPLP¹⁶⁴ amino acid sequence is involved in this function (Fig. 1.7) (Yang et al, 2003). As of yet, the exact role of multimerisation in Vif function and its active form within cells is still to be known.

1.13.6 Vif phosphorylation

HIV-1 Vif is phosphorylated *in vivo* and *in vitro* by cellular kinases. The phosphorylation of Vif was first identified by Yang et al through [³²P] orthophosphate labelling of the *in vivo* transfected and expressed Vif protein in HeLa cells. Subsequently they performed kinase assays by incubating CEM cell lysates with purified Vif protein in presence of [^{γ-32}P] ATP to show that Vif is also phosphorylated *in vitro*. Analysis of the [³²P]-labelled phosphoamino acids within the phosphorylated Vif protein pointed towards the involvement of serine/threonine residues in Vif phosphorylation (Yang et al, 1996).

To identify the Vif phosphorylation sites, the purified protein was phosphorylated *in vitro* with [^{γ-32}P] ATP in the presence of CEM cell lysate and subjected to protease digestion followed by radioactive peptide sequencing. Four major phosphorylation sites: T96, S144, T155 and T188, have been identified within the Vif sequence (Fig. 1.7) (Yang et al, 1996; Yang & Gabuzda, 1998).

Importantly, S144 is a part of the highly conserved ¹⁴⁴SLQYLA¹⁴⁹ motif (Section 1.12.4.3) and phosphorylation of Vif at this site is critical for its activity as demonstrated by the fact that replacement of this residue with alanine results in loss of Vif activity and also inhibits HIV-1 replication (Yang et al, 1996). Vif binding to Elongin C is also negatively regulated by the phosphorylation of S144 within the SOCS-Box motif (Mehle et al, 2004a). Elongin C is an important part of the E3 ubiquitin ligase complex required for the Vif-mediated degradation of A3G. Thus phosphorylation is important for productive HIV-1 infection but not A3G degradation. Thus the phosphorylated form of Vif may have some other as yet unknown functions independent from the degradation of A3G. Similarly, mutations of the T96 phosphorylation site result in inhibition of HIV-1 replication and significant loss of Vif activity (Yang & Gabuzda, 1998).

The cellular kinases responsible for the phosphorylation and regulation of Vif protein were found to be p44/42 Mitogen-activated protein kinases (MAPK) (also known as ERK-1 and ERK-2). ERK1 and ERK 2 are present in all cell types and play a role in cellular proliferation and differentiation in response to various mitogens, growth factors and cytokines. Thus there exists a direct link between MAPK and HIV-1 replication & infectivity and activation of MAPK by various mitogens and extracellular stimuli may contribute to activation of HIV-1 replication (Yang & Gabuzda, 1999).

Thus phosphorylation plays an important part in Vif function. However its complete role in the replication cycle of HIV-1 is yet to be fully understood.

1.13.7 The Vif-Pr55^{GAG} interaction

Vif has been shown to interact with the precursor of the virion structural proteins, Pr55^{GAG}. However, there are conflicting reports regarding the region of Vif involved in this interaction. Previous *in vitro* interaction studies suggested the carboxy-terminal 22 amino acids as being important for this interaction (Bouyac et al, 1997a) however recent *in vivo* studies point towards the N-terminal Arginine-rich region and the central area (residues 70-100) of the Vif sequence as being indispensable for the Vif-Pr55^{GAG} interaction (Syed & McCrae, 2009).

1.13.8 The Vif-genomic RNA interaction

Vif binds to HIV-1 genomic RNA *in vivo* (Dettenhofer et al, 2000; Khan et al, 2001) and *in vitro* (Dettenhofer et al, 2000; Henriët et al, 2005) and any mutation in the Vif sequence affecting its RNA function also reduces viral replication in non-permissive cells suggesting that RNA binding is crucial for Vif function (Khan et al, 2001; Zhang et al, 2000).

It has been reported that Vif binds to the genomic RNA in the cytoplasm of virus infected cells to form a 40S messenger ribonucleoprotein (mRNP) complex (Zhang et al, 2000). The same report also mentioned that Vif-RNA binding affinity decreases in the presence of Pr55^{GAG} while Vif does not exert any such effect on Gag-RNA binding. Recently it was shown that Vif preferentially and cooperatively binds to the 5' end of viral genomic RNA and is a part of a complex containing genomic RNA, Gag, Gag-Pol and HP68 (Henriët et al, 2005; Zimmerman et al, 2002). There is still considerable debate in the literature about whether Vif requires genomic RNA to mediate its encapsidation into the newly formed virus particles (Khan et al, 2001) or

whether the direct interaction between Vif and the NC domain of Pr55^{GAG} is sufficient for the incorporation of Vif into progeny virions (Bardy et al, 2001).

Vif has also been reported to promote the annealing of primer tRNA^{Lys3} to the primer binding site, favour genomic RNA dimerisation, decrease pausing of the RT and enhance first strand transfer during reverse transcription suggesting that Vif can operate as an RNA chaperone (Henriet et al, 2007).

1.13.9 Intravirion processing of Vif by HIV-1 Protease

Vif is packaged into the budding progeny virus particles where it is processed by the viral enzyme PR (Khan et al, 2002). The site of this processing has been mapped to position A150 within the Vif sequence (Fig. 1.7). Mutations in Vif which affect its PR processing also modulate viral infectivity indicating that processing of Vif is essential for its function (Khan et al, 2002).

1.13.10 Role of Vif in HIV core stability

Virions lacking Vif exhibit structural abnormalities in their nucleocapsid cores. The broad end of the cone shaped core is packed tightly with electron dense material but the opposite narrow end appears transparent (Borman et al, 1995; Bouyac et al, 1997b; Höglund et al, 1994; Ohagen & Gabuzda, 2000). In addition, viruses produced in the absence of Vif form nucleoprotein complexes that disassemble prematurely leading to the nucleolytic destruction of the nucleic acids within them (Simon & Malim, 1996). When virion cores derived from both Vif- and wild type HIV-1 are subjected to harsh conditions to mimic the environment at the time of uncoating of the core within a target cell during infection, viral cores derived in the absence of Vif are much less stable and release the core-associated proteins like p24, RT and p7 more readily compared to cores produced in the presence of Vif

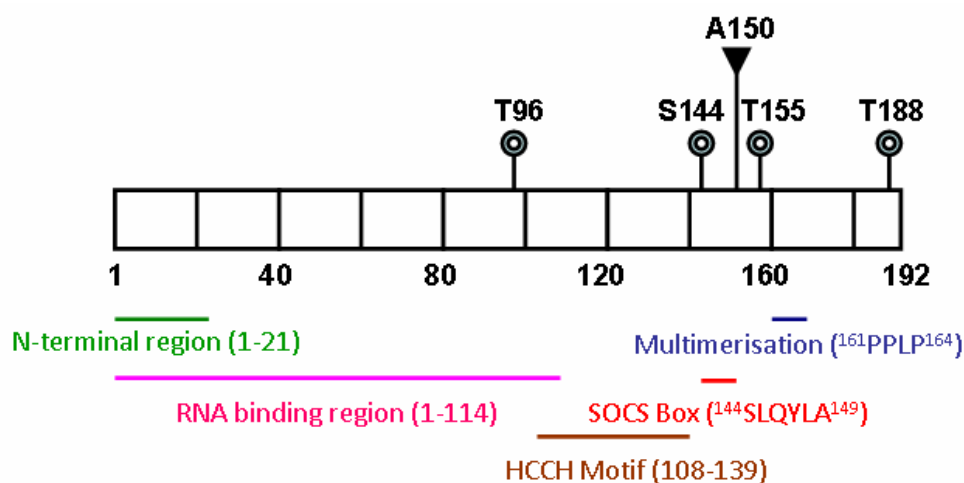


Figure 1.7 Schematic diagram of the Vif protein depicting its various functional domains:

The N-terminal tryptophan rich region (green) spanning 1-21 amino acids is involved in the suppression of A3G and A3F proteins (Tian et al, 2006). Amino acids 1-114 (pink) confer RNA binding activity to HIV-1 Vif (Khan et al, 2001; Zhang et al, 2000). The highly conserved HCCH motif (brown) spanning amino acids 108-139 consists of two His/Cys pairs and is involved in binding of Elongin C to Vif and the subsequent proteasomal degradation of A3 proteins (Luo et al, 2005; Yu et al, 2004). The SOCS-box motif from amino acids 144 to 149 (red) is also highly conserved and mediates the binding of Cullin 5 to Vif and thus playing an important role in A3 degradation (Yu et al, 2004). Residues 161-164 (blue) are involved in the multimerisation of Vif which is essential for Vif function (Yang et al, 2003). The four major phosphorylation sites for Vif have been depicted by the symbol \odot . These are T96, S144, T155 and T188 and play an important part in Vif function. Vif is processed by the viral enzyme protease and the site of PR processing has been mapped to A150 (Khan et al, 2002) and has been depicted by the symbol ∇ .

(Ohagen & Gabuzda, 2000). All these findings point towards a role for Vif in the stabilisation of the virion core thereby influencing the subsequent uncoating process to give mature viral nucleoprotein complexes and functional preintegration complexes capable of establishing a productive infection.

1.14 The APOBEC family of Cytidine Deaminases

1.14.1 Background

The APOBEC family of genes consists of Activation induced deaminase (AID) and APOBEC1 located on chromosome 12, APOBEC2 present on chromosome 6 and a series of 7 APOBEC3 genes found in a single gene cluster located on chromosome 22 (Goila-Gaur & Strebel, 2008; Jarmuz et al, 2002). APOBEC proteins are present only in vertebrates and exert their effects by cytidine deamination through conversion of cytidine residues to uracil inducing hypermutation in either DNA or RNA. While APOBEC1 acts on mRNA, APOBEC3 and AID act on single stranded DNA (Wedekind, 2003; Yoshikawa et al, 2002).

The earliest recognised family member is AID which is selectively expressed in activated B cell germinal centres. It plays an important role in the innate immune response by promoting antigen derived antibody diversification through deamination of the cytidine residues in immunoglobulin genes (Conticello, 2008). This leads to immunoglobulin gene diversification, somatic hypermutation and class-switch recombination events (Franca et al, 2006; Neuberger et al, 2003). Like AID, APOBEC2 also has a cytidine deaminase motif and is expressed mainly in cardiac

and smooth muscles. However the role it plays in these cells is yet to be understood (Franca et al, 2006).

APOBEC1 and APOBEC3 have developed later in evolutionary terms and are specific to mammals. APOBEC1 is expressed in the human small intestine and is responsible for ApoB pre-mRNA editing. It deaminates the cytidine at position 6666 within the ApoB mRNA to uracil thereby changing a glutamine codon within its sequence into a stop codon. This results in the formation of ApoB48, a truncated functional form of ApoB instead of ApoB100 which is the original form. ApoB48 is an important constituent of the chylomicrons, the very low density lipoproteins (VLDL) responsible for the transport of triglycerides from the intestine to various body tissues (Anant et al, 1995; Conticello, 2008; Yang et al, 2000).

The A3 family of proteins consists of 7 genes in humans and is discussed in more detail in section 1.14.2. It is interesting to note that while rodents express just one APOBEC3 gene in addition of APOBEC1, 2 and AID, humans and other non-human primates have 7 APOBEC3 genes. The mechanism of this expansion in humans and other primates remains unclear. It is believed that APOBEC genes first evolved to counteract the activity of endogenous retrotransposons and so prevent genetic instability and later expanded their repertoire to exogenous invading genetic elements as well. This could be one of the reasons why there was an expansion in the number of APOBEC3 genes in humans and other non-human primates (Franca et al, 2006).

1.14.2 APOBEC3 proteins

As indicated in section 1.13.2, the cellular anti-HIV factor counteracted by Vif was identified as belonging to the APOBEC3 (A3) family of proteins. The APOBEC3

family consists of APOBEC3A, APOBEC3B (A3B), APOBEC3C, APOBEC3DE (A3DE), APOBEC3F (A3F), APOBEC3G (A3G) and APOBEC3H. All these molecules act as cytidine deaminases and convert dC residue in DNA or RNA into dU thereby inducing hypermutation. Each member of the APOBEC3 family contains a characteristic domain structure. A short α -helical domain (H) is followed by a catalytic domain (CD). The CD is connected to a pseudocatalytic domain (PCD) by a linker (L) peptide (Fig. 1.8) (Jarmuz et al, 2002). In A3B, A3DE, A3G and A3F these domains are duplicated. Thus the domain organisation in these enzymes is H1-CD1-L1-PCD1-H2-CD2-L2-PCD2. Each CD contains a conserved motif H-X-E(X)₂₃₋₂₈-P-C(X)₂₋₄-C in which the Histidine and Cysteine residues coordinate Zn²⁺ ions and the Glutamic acid is involved in the proton shuttling process that occurs during the deamination reaction (Betts et al, 1994; Goila-Gaur & Strebel, 2008; Jarmuz et al, 2002).

1.14.3 Antiviral activities of various A3 proteins

While A3G is by far the most studied member of the APOBEC3 family, there are six other members of the A3 family, A3A, A3B, A3C, A3DE, A3F and A3H, which are also involved in the mutation and restriction of HIV-1 under different conditions.

However the most important antiretroviral A3 protein in terms of natural HIV-1 infection in humans other than A3G is A3F (Malim, 2009). It has almost 50% sequence similarity to A3G and also has a Vif-sensitive restriction phenotype (Liddament et al, 2004; Wiegand et al, 2004). It is interesting to note that the sequence elements in Vif required to overcome the inhibition of A3G and A3F are distinct from each other (Russell & Pathak, 2007; Simon et al, 2005). A3G and A3F are both expressed in CD4⁺ T cells, the natural host cells for HIV in humans, and the

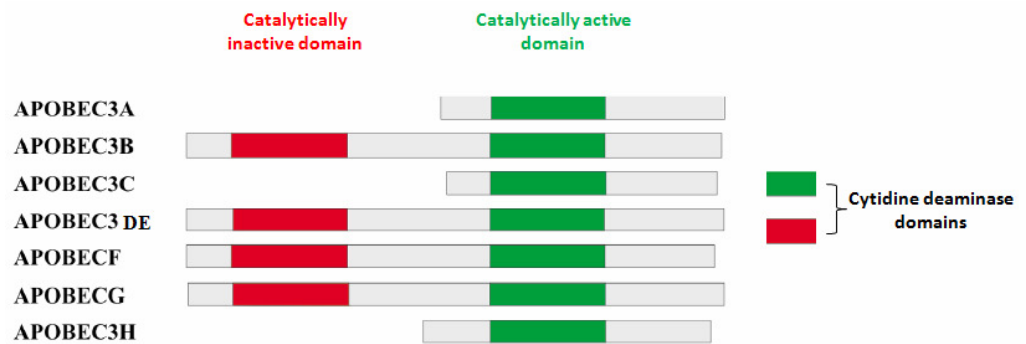


Figure 1.8 Domain organisation of APOBEC3 proteins: This figure depicts the domain structure of the members of the APOBEC3 family. All molecules of the family have a conserved cytidine deaminase domain in their structure. While 3B, 3DE, 3F and 3G have 2 copies of the domain within their coding sequence, 3A, 3C and 3H only have one copy. The two-domain enzymes have a catalytically active domain (Green) and a catalytically inactive domain (Red) (Figure modified from: Goila-Gaur *et al*, 2008).

Vif protein of HIV-1 has evolved to evade the antiretroviral effects of both these proteins allowing normal viral replication within these cells.

Among the remaining candidates, A3B has been shown to inhibit HIV-1 replication even in the presence of Vif but the experiments have been carried out in overexpression single cycle infectivity assays and not in a spreading infection system (Bishop et al, 2004; Doehle et al, 2005). Moreover, the level of expression of A3B in CD4⁺ T cells is very low and hence it is unlikely that it would have a major effect on the natural course of HIV-1 infection (Malim, 2009).

A3A, A3C, A3DE and A3H have all been shown to restrict HIV-1 infection modestly in a Vif-sensitive manner under various experimental conditions however the role played by these moieties in the natural course of HIV-1 infection is still under investigation (Bourara et al, 2007; Dang et al, 2006; Peng et al, 2007; Tan et al, 2009).

1.14.4 APOBEC3G and its effects on viral infectivity

1.14.4.1 Intracellular A3G complexes and antiviral activity

A3G also has the ability to bind RNA in addition to its well documented effects on single-stranded DNA (Chelico et al, 2006; Iwatani et al, 2006). In fact, the RNA binding function may be important for regulating its catalytic and antiviral activity as demonstrated from the finding that RNase treated A3G samples are catalytically more active (Chiu et al, 2005). Thus RNA may have a role to play in the formation of the cytoplasmic A3G High molecular mass (HMM) ribonucleoprotein (RNP) complexes observed in T-cells lines and activated CD4⁺ T-cells (Goila-Gaur et al, 2008; Kozak et al, 2006; Wang et al, 2007b). Activated CD4⁺ T-cells are more permissive to HIV-1 than resting PBMCs in which there is a post-entry block to viral

replication (Chiu et al, 2005). In activated CD4⁺ T-lymphocytes A3G is predominantly found in the HMM form while in resting CD4⁺ T-cells it is found in a low molecular mass or LMM form (Chiu et al, 2005). Thus there appears to be a correlation between antiviral activity and the cellular configuration of A3G (Goila-Gaur & Strebel, 2008). Analysis of the deaminase activity of A3G in transiently transfected 293T cells has revealed that HMM A3G was catalytically less active than LMM A3G but the activity of HMM A3G could be restored by RNase treatment (Chiu et al, 2005). Thus the current view is that HMM A3G is catalytically inactive while LMM A3G raises a post entry block to viral replication. It is important to note here that this post entry block to viral replication is in no way related to the Vif-sensitive restriction of HIV-1 which depends on the incorporation of A3G into budding virus particles and subsequent effect of A3G on viral DNA synthesis in the next cycle of viral replication.

1.14.4.2 Vif-sensitive restriction of HIV-1 by A3G

A broadly-accepted hypothesis to explain A3G inhibition of HIV-1 replication can be summarised as follows. In the absence of Vif, A3G from 'non-permissive' cells such as PBMCs, becomes incorporated into the assembling HIV virion through an interaction with the nucleocapsid (NC) region of Pr55^{GAG}. An early event in HIV replication is the reverse transcription of the RNA genome into DNA. A3G is proposed to target the cytidine residues present in the single-stranded DNA intermediate. By deamination, it converts them to uracil, resulting in large numbers of guanine to adenine transitions in the proviral DNA synthesised. This hypermutation of the viral genome leads to its inactivation and the resultant phenotype of greatly reduced yield of infectious virus (Mangeat et al, 2003). However, the presence of functional Vif is proposed to promote the prior degradation

of A3G by recruitment into an E3 ubiquitin ligase complex, resulting in the polyubiquitination of A3G and its rapid proteasome-dependent degradation (Yu et al, 2003). This Vif-induced degradation of A3G means that it is not available for incorporation into the maturing virus particles and so a normal yield of infectious virus is produced.

1.14.4.3 Deaminase-independent mechanisms of HIV-1 inhibition by A3G

It became widely accepted that the antiviral activity of A3G correlated broadly with its cytidine deaminase function. However recently there have been a number of reports dissociating antiviral activity from cytidine deaminase function. A3G was found to inhibit the process of HIV-1 reverse transcription (Iwatani et al, 2007). A different group has reported that A3G inhibited the annealing of tRNA^{Lys3} with viral RNA thereby interfering with the initiation of reverse transcription (Guo et al, 2006). A3G was also reported to inhibit the DNA strand transfer reaction during the reverse transcription process (Li et al, 2007). Another report suggested the involvement of A3G with the HIV-1 Integrase and its inhibition of integration and proviral DNA formation (Luo et al, 2007). However there is still considerable disagreement in the literature about whether or not the deaminase-independent effects of A3G are sufficient to explain its antiviral activity.

1.15 The role of Vif in A3G antagonism

Expression of Vif in cells containing A3G leads to a gradual reduction in the steady state levels of A3G while the A3G mRNA levels remain relatively constant. A3G is an inherently stable protein with a half life of >8 hr (Goila-Gaur & Strebel, 2008). In pulse/chase analyses, in the presence of Vif the half-life of A3G was found to be

between 5 min and 4 hrs depending on the experimental protocol used (Goila-Gaur & Strebel, 2008; Marin et al, 2003; Mehle et al, 2004b; Stopak et al, 2003).

As indicated in section 1.14.4.2, this reduction in A3G stability was found to be due to the Vif-induced degradation of A3G by the cellular ubiquitin-dependent proteasome system. Several domains in Vif are critical for this function. The highly conserved SLQ(Y/F)LA motif (referred to as SOCS box section 1.13.4) near the C-terminus is involved in its binding with Elongin C, a component of the E3 ubiquitin ligase complex (Mehle et al, 2004a; Mehle et al, 2004b; Yu et al, 2003; Yu et al, 2004). The HCCH domain (section 1.13.4) upstream of the SOCS motif is involved in the interaction with Cullin 5 (Luo et al, 2005; Mehle et al, 2004a; Yu et al, 2004). Thus the HCCH domain together with the SOCS box motif enable Vif to recruit the E3 ubiquitin ligase complex containing Cullin5, Elongin B, Elongin C and Rbx1 (Yu et al, 2003). Rbx1 complexes with A3G and speeds its polyubiquitination and subsequent degradation by the 26s proteasome (Yu et al, 2003).

Unfortunately, as further studies have been reported, this straightforward model of A3G inhibition and its Vif-mediated alleviation has become undermined by observations that cannot easily be accommodated by the simple model. These include:

- (i) A mutation in A3G that renders it resistant to Vif induced proteasomal degradation but whose inhibition of HIV can still be alleviated by Vif (Opi et al, 2007).
- (ii) Mutants of Vif that have lost the ability to induce A3G degradation but have retained the ability to block the inhibition of HIV replication by A3G (Yamashita et al).

- (iii) Mutants of A3G that appear to have lost their cytidine deaminase activity but nevertheless retain the ability to block replication of Vif deleted virus (Bishop et al, 2006; Iwatani et al, 2007).
- (iv) The severe impairment of virion nucleocapsid morphology and reverse transcriptase activity of Vif deleted (Δ Vif) virus produced from the first cycle of growth in non-permissive cells (Goncalves et al, 1996; Hoglund et al, 1994).
- (v) Fusion of A3G to a Vpr fragment allows it to escape the Vif blockade even though it continues to be degraded by Vif (Ao et al, 2008).

These studies are discussed in greater detail in Chapter 3. They have lead to a view emerging that whilst there has been rapid progress in trying to dissect Vif's role in overcoming the A3G based facet of the innate immune response against HIV-1, clear lacunae remain in detailed understanding of the mechanics of this process.

1.16 Hypothesis

The increasing number of reports showing that the mechanism of Vif-induced proteasomal degradation of A3G may not wholly explain its action in overcoming the A3G blockade to infectious virus production, suggest that some other mechanism may come into play in the Vif mediated exclusion of A3G from the HIV virion. It is known that both Vif and A3G proteins interact with Pr55^{GAG} of HIV allowing them to be specifically incorporated into the core of the budding progeny virions (Alce & Popik, 2004; Bouyac et al, 1997a). Hence the possibility exists that Vif actually competes with A3G for Pr55^{GAG} binding and excludes it from the budding virion core.

Therefore the central hypothesis that this study sets out to investigate is that a competition exists between Vif and A3G proteins for Pr55^{GAG} binding whereby Vif outcompetes A3G resulting in the exclusion of the inhibitor from the budding progeny virions and prevention of its harmful effects in the next cycle of virus infection, as an alternative mechanism of Vif in overcoming the A3G block to HIV replication.

1.17 Aims

To understand the dynamics between Vif and A3G in greater detail and obtain evidence to support the hypothesis outlined above the following were the experimental objectives of this study:

- (i) To determine whether Vif and A3G interact at the same site on Pr55^{GAG}. The simplest model of competition between these two proteins would involve both of them interacting at the same site and competing with each other for Pr55^{GAG} binding.
- (ii) After showing that both Vif and A3G interact at the same site on Pr55^{GAG}, the second objective was to demonstrate the competition between Vif and A3G for Pr55^{GAG} binding resulting in the displacement and exclusion of A3G by Vif.
- (iii) To study the sub-cellular localisation of Vif, A3G and Pr55^{GAG} and to find out if co-expression of Pr55^{GAG} with Vif/A3G results in a change in the localisation pattern of Vif and/or A3G.

CHAPTER 2

Materials and Methods

2.1 Suppliers

General chemicals were obtained from BDH Laboratory Supplies, Fisher Scientific (Loughborough, Leicestershire), Merck Ltd. (Poole, Dorset) and Sigma-Aldrich Company Ltd. (Gillingham, Dorset) and were of molecular biology, analytical or blotting grade.

Equipment and other reagents (unless otherwise stated) were obtained from the following suppliers:

Bio-Rad Laboratories (Hemel Hempstead, Hertfordshire)

Ammonium persulphate, TEMED, sodium dodecyl sulphate (SDS).

Bio sera (East Sussex, U.K)

Foetal Calf Serum (FCS).

Fermentas UAB (Vilnius, Lithuania)

Prestained Protein Ladder 10-180 kDa. Restriction and modification enzymes.

GE Healthcare, Amersham Biosciences (Amersham, Buckinghamshire)

HybondTM Nitrocellulose membranes, ECLTM Advance Western Blotting Detection Kit

Invitrogen Corporation, R & D Systems Ltd., (Abingdon, Oxfordshire)

TA CloningTM System, restriction and modification enzymes, *Taq* DNA polymerase, 1 Kbp DNA ladder, LipofectamineTM 2000 Reagent.

New England Biolabs (UK) Ltd., (Hitchin, Hertfordshire)

Restriction and Modification enzymes.

Qiagen Ltd., (Crawley, West Sussex)

QIA quick Gel extraction kit.

Roche Diagnostics Ltd., (Lewis, East Sussex)

CAT ELISA kit.

Sigma-Aldrich Company Ltd., (Gillingham, Dorset)

Butanol (molecular biology grade), kanamycin, ampicillin, SigmaSpin™ Maxiprep kit

Whatman International Ltd., (Maidstone, Kent)

Filter paper Grade 5 (70mm)

National Diagnostics Ltd., (Hessle, North Humberside)

ProtoGel 30%

Department of Biological Sciences, University of Warwick – Media Preparation

LB broth, LB agar, DMEM, Versene, trypsin, phosphate buffered saline (PBS), 10X Tris-Boric acid-EDTA (TBE), 10X SDS PAGE running buffer, 10X protein transfer buffer.

2.2 Materials

2.2.1 Standard buffers and solutions

Luria-Bertani Broth (LB): 1% (w/v) bactotryptone, 1% (w/v) NaCl, 0.5% (w/v) yeast extract.

LB agar: LB Broth containing 1.5% (w/v) bacto-agar.

Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM Na₂HPO₄, 1.4 mM KH₂PO₄

2X Sample buffer: 0.2% (w/v) SDS, 1% (v/v) glycerol, 50 mM Tris-HCl buffer (pH6.8), bromophenol blue.

1X SDS PAGE loading buffer: 50% (v/v) 2 x sample buffer, 100 mM dithiothreitol (DTT)

SDS PAGE running buffer: 0.05M Trisma base, 0.384M glycine, 0.1% (w/v) SDS

Tris-HCl buffered saline (TBS): 50 mM Tris.HCl, 150 mM NaCl, pH 8.0

TBE: 90mM Tris base, 90mM boric acid, 1mM EDTA, pH8.0.

10X Agarose gel loading buffer: 50% (v/v) glycerol, 0.01% (w/v) bromophenol blue, 0.01% (w/v) xylene cyanol, 2% TBE (v/v).

10X Protein transfer buffer: 1 L: 30.3 g Trizma base (= 0.25 M) ,144 g Glycine (= 1.92), pH should be 8.3; do not adjust.

TfbI: 10 mM RbCl₂, 10 mM CaCl₂, 30 mM potassium acetate, 50 mM MnCl₂, 15% (v/v) glycerol, pH 5.8.

TfbII: 10 mM RbCl₂, 75 mM CaCl₂, 10 mM 3[N-morpholino] propanesulphonic acid (MOPS), 15% (v/v) glycerol, pH 6.5.

1X HBS: 1L : 5g HEPES (Acid), 8g NaCl, 1g Dextrose, 3.7g KCl, 10ml Na₂HPO₄(7H₂O)

TSE: 20 mM Tris HCl pH 7.5, 150 mM NaCl, 1 mM EDTA

Stripping Buffer: 2% (w/v) SDS, 62.5mM Tris-HCl pH 6.8, 100mM β-mercaptoethanol

2.2.2 Bacterial Strain

INVαF' (Invitrogen)

Genotype: F' *endA1 recA1 hsdR17* (rk-, mk+) *supE44 thi-1 gyrA96 relA1* φ80*lacZ*ΔM15

Δ(*lacZYA-argF*)U169 λ-

2.2.3 Cell lines

a) COS-1

Description: SV40 transformed African Green Monkey Kidney cells (supplied by Prof. M. McCrae, University of Warwick)

Culture Medium: DMEM + 10% FCS (v/v)

b) 293T

Description: SV40 transformed Human Embryonic Kidney cells (supplied by Prof. M. McCrae, University of Warwick)

Culture Medium: DMEM + 10% FCS (v/v)

2.2.4 Monoclonal antibodies

- a) **Anti-HA** antibody, produced in mouse (Sigma-Aldrich Cat. No. H9658) used at 1:10,000 dilution for western blots and 1:250 dilution for immunofluorescence procedures.
- b) Mouse **anti-Vif** monoclonal antibody (Cat. No. 2745) obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: from Transgene used at 1:250 dilution.
- c) **mAb to p24** (ARP 454) obtained from the Programme EVA Centre for AIDS Reagents, NIBSC, UK and was donated by Drs. Ferns and Tedder, and DAKO Ltd UK used at 1:3000 dilution.
- d) **Anti-Mouse IgG (γ -chain specific)–Alkaline Phosphatase** antibody produced in goat (Sigma-Aldrich Cat. No. A3438) used at 1:25,000 dilution.
- e) **Anti-mouse IgG (whole molecule)–Peroxidase** antibody, produced in Goat (Sigma-Aldrich Cat. No. A4416) used at 1:5000 dilution.

- f) **Anti-Rabbit IgG (whole molecule)–Peroxidase** antibody produced in goat (Sigma-Aldrich Cat. No. A6154) used at 1:100,000 dilution.
- g) **Alexa Fluor® 594 goat anti-rabbit IgG (H+L)** antibody (Invitrogen Cat. No. A-11012) used at 1:3000 dilution.
- h) **Alexa Fluor® 594 goat anti-rabbit IgG (H+L)** antibody (Invitrogen Cat. No. A-11005) used at 1:500 dilution.

2.2.5 Antisera

Rabbit HIV-1_{HXB2} Vif Antiserum obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: from Dr. Dana Gabuzda used at 1:1000 dilution for western blots and 1:200 dilution for immunofluorescence detection.

2.2.6 Primers

The oligonucleotide primers utilised in this study are shown in Table 2.2.1. All the primers were synthesised and supplied by Invitrogen Corporation unless stated otherwise.

Table 2.2.1 Oligonucleotide primers used in this study

Primer Name	Primer Sequence	Used for
NCDel	5'GGC AAT TTT AGG AAC CAA AGA AAG3'	NC Region of Pr55 ^{GAG} from amino acid 4 to 11; Position: 1476 of the HIV Genome
NCReverse	5'TTG TCT ATC GGC TCC	Amplification of NC Region of

	TGC TTC TGA3'	Pr55 ^{GAG} ; Position: 1777 of the HIV Genome
NUCSeq	5'AGC ATT GGG ACC AGC GGC TAC ACT3'	Sequencing Primer for NC Region of Pr55 ^{GAG} ; Position: 1340 of the HIV Genome
GagHpaF	5'CCA AAG AAA GAT TGT TAA CTG TTT CAA TTG TGG C3'	Creation of HpaI site at amino acid 13 on NC Region of Pr55 ^{GAG} ; Position: 1490 of the HIV Genome
GagHpaR	5'GCC ACA ATT GAA ACA GTT AAC AAT CTT TCT TTG G3'	Creation of HpaI site at amino acid 13 on NC Region of Pr55 ^{GAG} ; Position: 1523 of the HIV Genome
EcoGagF	5'CTT GGT GAA TTC ATG GGT GCG AGA GCG 3'	Amplification of Gag from its origin along the establishment of an EcoRI site at its N terminal end; Position: 336 of the HIV Genome
HpaIGagR	5'GCG CGT GTT AAC TCT CTG CAT CAT TAT GG3'	Pr55 ^{GAG} amplification from amino acid 3 of the NC in reverse direction along with establishment of a HpaI site at the end; Position: 1475 of the HIV Genome

1551GagMu tF	5' CA GCC AGA AAT TGC AGA GCT CCT GAG GAA AAG GGC TGT TGG AAA TG 3'	Forward primer for site directed mutagenesis of Pr55 ^{GAG} to replace two basic amino acids with two acidic ones in the Basic Linker region of the NC
1551GagMu tR	5' CA TTT CCA ACA GCC CTT TTC CTC AGG AGC TCT GCA ATT TCT GGC TG 3'	Reverse primer for site directed mutagenesis of Pr55 ^{GAG} to replace two basic amino acids with two acidic ones in the Basic Linker region of the NC
GagRSeq	5'GTC TGA AGG GAT GGT TGT AG3'	Reverse sequencing primer for the N-Terminal region of Pr55 ^{GAG} along with the start codon; Position: 544 of the HIV Genome
A3GRSeq	5'GCT GAA CCA GTG GAA GAA TC3'	Reverse sequencing primer for Apobec3G N-Terminal terminal region along with the start codon; Position: 456 of the APOBEC3G sequence
pMSeq	5'AAG TGC GAC ATC ATC ATC GG3'	Sequencing primer for pM vector for checking if the inserted gene sequence is in

		frame with the GAL4-BD; Position: 793 of the pM vector sequence
pVPSeq	5' GAT GTT TAC CGA TGC CCT TG3'	Sequencing primer for pVP vector for checking if the inserted gene sequence is in frame with the VP16-AD; Position: 612 of the pVP16 vector sequence
pcDNASeq	5'CCA CTG CTT ACT GGC TTA TC3'	Sequencing primer for pcDNA 3.1 vector for checking the sequence and alignment of the inserted gene; Position: 838 of the pcDNA3.1 vector sequence
DrC97AF	5'ATA TCC TGG AGC CCC GCT ACA AAG TGT ACA AGG 3'	Forward primer for site-directed mutagenesis of A3G-HA replacing Cysteine at position 97 with Alanine
DrC97AR	5'CCT TGT ACA CTT TGT AGC GGG GCT CCA GGA TAT 3'	Reverse primer for site-directed mutagenesis of A3G-HA replacing Cysteine at position 97 with Alanine
EcoRIHAF	5'GAA TTC GCC ACC ATG TAC CCA TAC GAT 3'	Forward primer for the amplification of the 3xHA to tag it to the N-terminal end of

		Vif
EcoRIHAR	5' GAA TTC AGC GTA ATC TGG AAC GTC ATA 3'	Reverse primer for the amplification of the 3xHA to tag it to the N-terminal end of Vif

2.3 Molecular biology techniques

2.3.1 Agarose gel electrophoresis

DNA or cDNA samples were electrophoresed on 0.8 to 1.3 % Agarose gels containing 1µg/ml of Ethidium Bromide in 1X TBE buffer. 1/10 volume of 10X TBE agarose gel loading buffer was added to the samples and the 1kB DNA ladder before loading into preformed wells in the agarose gel. The samples were electrophoresed at 70-100 mA for 60-90 minutes. The bands were visualised using the Bio-Rad Gel Doc XR system and analysed using the Quantity One 1D gel analysis software.

2.3.2 Quantification of DNA

Concentration of DNA solutions was determined by comparing an unknown DNA sample band on an agarose gel with a known quantity of 1Kb DNA ladder. The 1636 bp band on the Invitrogen 1Kb DNA ladder was used as the reference. When 1µg of ladder was loaded per lane the 1636 bp band contains approximately 100ng of DNA.

DNA concentrations were also measured using a NanoDrop ND-1000 V3.2.1 (Thermo Scientific) at A_{260} where an absorbance of 1.0 corresponded to 50 µg/ml of DNA. The quality of DNA was estimated by using the A_{260}/A_{280} ratio where a value ≥ 1.8 indicated a pure sample.

2.3.3 Ethanol precipitation of nucleic acids

DNA was precipitated from aqueous solutions containing 0.2M LiCl and at least 2.5 volumes of ice cold 100% ethanol. The solution was kept at -70°C for 1 hour or at -20°C overnight. The precipitated DNA was pelleted by centrifugation at 12000 rpm for 20 minutes. The supernatant was decanted and the pellet washed with 80% ice cold ethanol and centrifuged at 12000 rpm for a further 10 minutes. The supernatant was again discarded post-centrifugation and the pellet dried in vacuum for 30 minutes. The dried pellet was resuspended in sterile water.

2.3.4 Phenol extraction of nucleic acids

Nucleic acid solutions were mixed with 2 volumes of Phenol/50mM Tris.HCl thoroughly for 1 minute. The samples were centrifuged briefly to separate the phenol and aqueous phases. After discarding the separated phenol, the aqueous solution was mixed with 2-3 volumes of diethyl-ether for 1 minute. Ether extraction was carried out 3 times until the aqueous solution became clear. The excess ether was removed in a stream of nitrogen gas for 1 minute.

2.3.5 Polymerase Chain Reactions

Taq DNA Polymerase was used for most routine PCR applications. However more demanding applications such as site-directed mutagenesis were performed using Platinum Pfx DNA Polymerase which has a higher fidelity, specificity and yield than Taq polymerase.

Standard PCR reactions were carried out in a final volume of 25-50 µl. For a 25 µl PCR reaction the following were mixed in a 500 µl eppendorf tube. For larger

reaction volumes, constituents were added in the same proportions as indicated below:

For PCR using Taq DNA Polymerase:

1µl of 50mM MgCl₂

2.5µl of 10X PCR Buffer {200 mM Tris-HCl (pH 8.4), 500 mM KCl}

0.5µl of Invitrogen Taq DNA Polymerase (5U/µl)

1 µl of 5mM dNTP mixture

1.5 µl of each Primer (100ng/ µl)

1 µl of DNA template (100ng/ µl)

The mixture was made up to a final reaction volume of 25 µl by adding sterile water.

Reaction conditions: 25 cycles of 94 °C for 1 min; 60 °C for 1 min; 72 °C for 2-4 min (extension time dependent on the size of the template to be amplified-1min/Kb) using a PeqLab Primus 25 advanced Thermocycler.

For PCR using Platinum Pfx DNA Polymerase:

0.5 µl of 50 mM MgSO₄

2.5 µl of 10X Pfx Amplification Buffer

1.25 µl of 10X PCR Enhancer Solution

0.2 µl of Platinum Pfx DNA Polymerase (2.5U/ µl)

1 µl of 5mM dNTP mixture

1.5 µl of each Primer (100ng/ µl)

1 µl of DNA template (100ng/ µl)

The mixture was made up to a final reaction volume of 25 µl by adding sterile water.

Reaction conditions: 94 °C for 2 min; followed by 25 Cycles of 94 °C for 30 Sec; 55 °C for 30 Sec; 72 °C for 5-8 min (extension time dependent on the size of the

template to be amplified-1min/Kb) using a PeqLab Primus 25 advanced Thermocycler.

2.3.6 Restriction enzyme digests

Restriction enzyme digests were performed according to the manufacturers' recommendations for the specific restriction enzymes. The incubation period used for most enzymes was 37 °C for 1 hour (unless otherwise mentioned). Digested DNA was electrophoresed on an agarose gel to confirm if the enzyme digest had worked.

2.3.7 DNA extraction from an agarose gel

Restriction enzyme digested DNA was electrophoresed on 0.9 to 1.3% agarose gel. The desired DNA band was cut from the gel slice and eluted using a QIA quick Gel extraction kit (Qiagen Ltd.) according to the manufacturer's instructions.

2.3.8 Dephosphorylation of plasmid DNA

Linearised plasmid DNA was subjected to dephosphorylation at its 5' ends by using Shrimp Alkaline Phosphatase (SAP) (Fermentas) to prevent self-ligation of the vector and increase the efficiency of its ligation with the insert being used. 4 µl of SAP (1U/ µl) was employed together with 3 µl of 10X SAP Reaction Buffer in a total volume of 30 µl. The samples were incubated overnight at 37 °C. The SAP enzyme in the samples was heat inactivated at 65 °C for 20 minutes prior to using the DNA in ligation reactions.

2.3.9 Ligation of DNA

For all ligation reactions, T4 DNA Ligase (Invitrogen) was used together with 5X Ligase Buffer. For ligations of insert into a linearised plasmid, the digested vector

was dephosphorylated with Shrimp Alkaline Phosphatase (Fermentas) prior to setting up the ligation.

Most ligation reactions were performed in a total volume of 20 µl. 4 µl of T4 DNA Ligase was used and 4 µl of 5X Ligase Buffer was added. Insert and vector were used in a molar ratio of 3:1. The amount of insert DNA used for ligation was calculated as follows:

$$\text{Insert (ng)} = \text{Vector (ng)} \times \{ \text{Insert (Kb)} / \text{Vector (Kb)} \} \times 3$$

The samples set-up for ligation were incubated at 16 °C for at least 16 hours. Post-incubation, chemically competent INVαF' *E. coli* were transformed with the ligated samples by the heat shock method of chemical transformation (section 2.4.3).

2.3.10 Sequencing

All ligated/mutated plasmid constructs were sequenced to check for in frame junctions/ orientation and to check the sequence for the introduction of any adventitious errors by the DNA polymerase. The DNA sequencing was carried out at the Molecular Biology Sequencing Service, Department of Biological Sciences, University of Warwick, using Big Dye Terminator Version 3.1 Chemistry and run on a 3100 Genetic Analyser (Applied Biosystems). The sequencing data was analysed using Chromas Lite 2.0 or Clone Manager, SciEd Central, v7.04.

2.4 Bacteriology techniques

2.4.1 Bacterial culture

E. coli cells were cultured in LB Broth overnight in an incubator-shaker at 37 °C shaking vigorously at 200-250 rpm. Chemically transformed bacterial cultures were either plated onto LB Agar plates or allowed to grow in LB Broth with appropriate antibiotics and incubated overnight at 37 °C. The transformed cells were stored for future use as 50% Glycerol (v/v) stocks.

2.4.2 Preparation of competent cells for chemical transformation

2ml of a fresh overnight culture of the INVαF' strain of *E. coli* was added to 100ml of LB broth prewarmed to 37 °C. The culture was allowed to grow to an OD₆₀₀ of 0.39. After the culture had reached the desired optical density, it was placed on ice for 5 minutes and pelleted by centrifugation at 6000rpm for 10 minutes at 4 °C. The pellet was resuspended in 40ml of TfbI, and re-centrifuged at 6000 rpm for 10 minutes at 4 °C. The resultant supernatant was discarded and the pellet was dissolved in 4 ml of Tfb II and incubated on ice for 2 hours. The resuspended competent cells were then separated into aliquots of 200 µl and stored at -80 °C for up to 8 weeks before use.

2.4.3 Chemical transformation of competent cells

Competent cells stored at -80 °C were thawed on ice and mixed with the ligation products (section 2.3.9). The resultant mixture was allowed to incubate for 30 minutes on ice, before the cells were heat shocked at 42 °C for 50 seconds and quickly placed back on ice. 500 µl of warm LB broth was added to the cells and they

were grown in an incubator-shaker at 37 °C and 200-250rpm for 2 hours. After 2 hours, the culture was plated onto LB agar plates with appropriate antibiotics and grown overnight at 37 °C.

2.4.4 Small-scale preparation of plasmid DNA from bacteria

Bacterial colonies from section 2.4.3 were picked from antibiotic-agar plates and inoculated into 5ml of LB broth with the appropriate antibiotic. The inoculated broth was cultured overnight at 37 °C in an incubator-shaker at 200 to 250 rpm. For extraction of plasmid DNA, 1.5 ml of the overnight culture was used. The DNA was extracted using a QIAprep Spin Miniprep Kit (Qiagen) following the manufacturer's protocol.

2.4.5 Large-scale preparation of plasmid DNA from bacteria

5ml of a fresh overnight culture of transformed bacteria carrying the desired plasmid from section 2.4.4 was added to 200 ml of LB broth and allowed to incubate overnight at 37 °C in an incubator-shaker at 200-250 rpm. For large scale extraction of plasmid DNA, the GenElute™ Maxiprep kit (Sigma-Aldrich) was used according to the manufacturer's instructions.

2.5 Basic techniques in mammalian tissue culture

2.5.1 Maintaining cells in culture

COS-1 and 293T cells were cultured in sterile tissue culture flasks at 37°C in a 5% CO₂ atmosphere. DMEM containing 10% Fetal Calf Serum (FCS) (v/v) was used as the culture medium for passage of both cell lines. Prior to passage, the cells were first

washed twice with Versene (0.02% EDTA in PBS). After these washes, cells were incubated in the presence of Trypsin solution (0.02% EDTA containing 0.25mg/ml Trypsin) for 2-3 minutes to detach adherent cells from the flask surface. The cell suspension was centrifuged at 1300rpm for 3 minutes to pellet the cells. The pelleted cells were resuspended in DMEM with FCS and plated at the required density.

2.5.2 Storage of Cell lines

Cells were grown to confluency, washed with versene and trypsinised as detailed in section 2.5.1. Post-trypsinisation, the cells were pelleted at 1300 rpm for 3 minutes. The resulting pellet was resuspended in culture medium. The concentration of cells/ml recovered was determined using a Neubauer's haemocytometer. If N was the total number of cells observed under the light microscope in the 4 peripheral squares, then,

$$\text{No. of cells/ ml of culture medium} = N/4 \times 10^4$$

After calculating the concentration, the cells were re-centrifuged at 1300 rpm for 3 minutes and resuspended in 90% FCS and 10% DMSO at a concentration of 10^7 cells/ml. Aliquots of 1 ml were transferred to cryogenic vials and stored at -20°C for 1 day, -70°C for the 2nd day and then transferred to liquid nitrogen for long term storage.

2.5.3 Lipofectamine transfection protocol for cells

COS-1/293T cells were transfected using Lipofectamine 2000 (Invitrogen) as the transfection reagent. One day prior to the transfection 10^6 cells per well were seeded in 12-well tissue culture dishes. The cells were allowed to grow overnight until they became ~85% confluent (COS-1 cells) or ~75% confluent (293T cells) at 37°C in a 5% CO₂ atmosphere and then transfected. The DNA to be transfected was diluted in

250 µl of serum-free DMEM in a 1.5 ml eppendorf tube. Lipofectamine 2000 was gently mixed before use and diluted separately in 250 µl of serum-free DMEM. A Lipofectamine (µl) / DNA (µg) ratio of 1:1.5 was used. After 5 minute incubation, the diluted DNA and diluted Lipofectamine were combined and mixed gently. The DNA-Lipofectamine mixture was allowed to incubate for 30 minutes. Culture medium was removed from cells and replaced with the DNA-Lipofectamine mixture. The cells were allowed to incubate with the serum-free mixture for 4 hours at 37°C. After 4 hours, the DNA-Lipofectamine mixture was replaced with 2 ml of fresh DMEM with 10% FCS + pen/strep. The transfected cells were incubated for 48-72 hours before being subjected to analysis.

2.5.4 Calcium phosphate transfection protocol for 293T cells

293T cells were transfected using Calcium phosphate as the transfection reagent. A day before transfection, a confluent 175 cm² flask of cells was split using a 1:15 ratio and seeded in 10 cm tissue culture dishes with 10ml of DMEM+ 10%FCS. After overnight incubation when the monolayer had reached 70-80% confluency, the media in each plate were replaced with fresh DMEM with 10% FCS & pen/strep and then transfected. The transfections were set up using 30 µg of total DNA. For each transfection 0.5ml of 1X HBS was added to a 1.5ml eppendorf tube. Appropriate DNAs were added to each tube and the contents were mixed thoroughly. 30µl of 2.5M CaCl₂ was added to each tube and mixed immediately. The mixture was allowed to sit for 30 minutes. The transfection mix was then added to the cells in a drop-wise fashion simultaneously rocking the tissue culture dishes back and forth. The transfected cells were allowed to be incubated at 37°C in a 5% CO₂ atmosphere. The following day the media in all dishes was replaced with 10ml of fresh DMEM

with 10% FCS. The cells were typically incubated for 48-72 hours before being subjected to further analysis.

2.6 Protein biochemistry techniques

2.6.1 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The Bio-Rad Mini-PROTEAN 3 system was used for all routine SDS-PAGE applications. The composition of the stacking gel and the resolving gel used in this study is shown in table 2.6.1 below:

Table 2.6.1 Composition of the stacking and resolving gel used in SDS-PAGE

(A) 12.5 % Resolving Gel

Components	Volume used
30% Acrylamide; 0.8%N'N'-bis-methylene-acrylamide	4.167
1.5M Tris-HCl pH 8.3	2.5
10% SDS	0.1
H ₂ O	3.178
Ammonium persulphate	0.05
TEMED	0.005

Total Volume = 10 ml

(B) 3% Stacking Gel

Components	Volume used
30% Acrylamide; 0.8%N’N’-bis-methylene-acrylamide	1.0
1.5M Tris-HCl pH 6.8	0.8
10% SDS	0.1
H ₂ O	8.0
Ammonium persulphate	0.1
TEMED	0.01

Total Volume = 10 ml

Protein samples suspended in 1X SDS-PAGE loading buffer were incubated in a boiling water bath for 5 minutes and then centrifuged at 13,200 rpm for 30 seconds. The samples were then loaded onto the gel and electrophoresed at 150V for 80 minutes. A prestained protein ladder (10-180 kDa; Fermentas) was used to determine the size of the proteins being analysed.

2.6.2 Western Blotting

After fractionation on SDS-PAGE gels, proteins were transferred onto ECL Hybond nitrocellulose membranes (GE Amersham) using a transblot apparatus (Bio-Rad) with 1X transfer buffer containing 20% methanol (v/v) either at 0.08 A for 16 hours or at 0.35 A for 1.5 hours at 4°C. Blotted proteins were detected either colorimetrically or by chemiluminescence.

2.6.3 Colorimetric detection of proteins on a Western Blot

Anti-mouse alkaline phosphatase conjugated antibody (Sigma-Aldrich A3438) was used as a secondary antibody to detect proteins colorimetrically by employing BCIP/NBT Phosphatase substrate tablets (Sigma-Aldrich B5655) according to the manufacturer's instructions.

2.6.4 Chemiluminescence detection of proteins on a Western Blot

Anti-mouse IgG (whole molecule)-Peroxidase antibody, produced in Goat (Sigma-Aldrich A4416) was used as the secondary antibody for chemiluminescence detection of proteins by employing the ECL-Chemiluminescence Detection Kit (GE Healthcare Life Sciences) and the blot analysed using the Bio-Rad ChemiDoc XRS imaging system according to the manufacturer's instructions.

2.6.5 Reprobing the same blot with different antibodies

After detection of one protein in a blot, if the same blot needed to be probed for a different protein, stripping buffer (section 2.2.1) was used. After the first detection, the blot was incubated in a 50⁰ C water bath for 15 minutes in presence of stripping buffer. Post-incubation, the blot was given 3×15 minute washes with TBS. Subsequently the blot was then probed again with antibody for the second protein.

2.7 Mammalian two-hybrid assay

COS-1 cells were transfected with pM and pVP16 plasmid constructs together with either CAT or GFP reporter plasmid using the standard Lipofectamine transfection protocol (section 2.5.3). The transfected cells were allowed to incubate at 37°C in a

5% CO₂ atmosphere for 48-72 hours. GFP reporter expression was monitored in live cells 48 hours post-transfection using a UV light microscope. Subsequently the GFP expression within transfected cells was analysed using the ImageJ image analysis software (described in detail in section 2.10). The GFP reporter expression data was further confirmed by quantitatively assaying the CAT reporter expression using a CAT ELISA kit (Roche) and transfected cell extracts made 72 hours post-transfection by following the manufacturer's instructions.

2.8 Sucrose gradient analysis of GAG virus-like particles (VLPs)

293T cells were transfected with a Pr55^{GAG} expression plasmid (pGAG-EGFP) using the standard Calcium phosphate transfection protocol (section 2.5.4). GAG-EGFP chimeric protein expression was observed in live cells using the UV light microscope 48 hours post-transfection. Subsequently, cell culture supernatants containing VLPs from transfected 293T cells were harvested at 72 hours post-transfection, clarified by centrifugation at 1300 rpm for 3 minutes and filtered through a 0.45µm pore-sized filter. The filtered supernatant was centrifuged through a 20% sucrose cushion in a Beckman SW-28 rotor at 100,000 g for 2 hrs at 4°C to pellet the VLPs. The pellets were resuspended in Phosphate Buffered Saline (PBS). GAG VLPs resuspended in PBS were overlaid onto linear 20-60% sucrose gradients and centrifuged in a Beckman SW40Ti rotor for 16 hrs at 4°C. After ultracentrifugation, equal fractions were collected from the bottom of the gradient, fraction 1 being the lowermost and fraction 14 being the uppermost with 'p' being the resuspended pellet. Proteins were later precipitated from the collected fractions by treating with 10% Trichloroacetic acid (TCA). The TCA precipitates were collected by centrifugation at 13,200 rpm for

15 min. The supernatant from the centrifuged samples was discarded and pellets were resuspended in SDS-sample buffer, 8M Urea and 2M Sodium hydroxide (NaOH) for analysis by SDS-PAGE as detailed in section 2.6. The VLP proteins incorporated into the nitrocellulose membrane were probed as described in section 2.2.4 and analysed using chemiluminescence (section 2.6.4).

2.9 Immunofluorescence imaging of transfected cells by confocal microscope

293T cells were used for transfection and subsequent immunofluorescence procedures. Cover slips were placed at the bottom of the 12 well tissue culture plates used for transfection. Subsequently the cells were plated onto the wells containing cover slips in such a way that the monolayer growth occurred on the cover slip surface. The cells were allowed to grow overnight until they became ~75% confluent. Later, they were transfected using the standard Lipofectamine transfection protocol mentioned in section 2.5.3.

2.9.1 Fixing of cells on coverslips

48 hours post-transfection, the cells were washed twice with 0.5 ml PBS and incubated with 0.5 ml of 10% (w/v) formaldehyde in PBS for 10 minutes. Post-incubation, the formaldehyde was discarded and the cells were washed again with PBS. Subsequently the cells were incubated with 0.5 ml 0.5% NP40 in PBS for 10 minutes to permeabilise them. The cells were again washed with PBS after this incubation. If the proteins to be detected were tagged with GFP, then the coverslips were directly washed in the presence of nuclear stain DAPI and imaged under the

confocal microscope. However, if the proteins were not GFP tagged, they were probed with primary and secondary antibody as described below before analysis.

2.9.2 Staining

After the last PBS wash, the cells were blocked for 1 hour in 0.5ml of 10mg/ml BSA in PBS. Later the cover slips were washed thrice with PBS. 0.25ml of primary antibody diluted in 1% BSA in PBS was then added to the wells containing cover slips using the optimised concentration and left to incubate for 1 hour. After 1 hour, the cells were again washed thrice with PBS. 0.25 ml secondary antibody diluted in 1% BSA in PBS was later added **in dark** to the wells and the cells were allowed to incubate for 1 hour. The cover slips were later washed in dark for 3 times with PBS. The final PBS wash was performed in the presence of nuclear stain DAPI at 1µg/ml (in PBS) and allowed to incubate for 5 minutes in dark. The cover slips were later removed from wells after the DAPI wash and dried on filter paper in dark. The dried cover slips were inverted onto a small amount of mounting medium (Vectorshield) on a slide and the edges of the cover slips were sealed with nail varnish. Finally the cover slips mounted on the slides were used for imaging using Leica TCS SP5 confocal microscope.

2.10 Image analysis using ImageJ software

The fluorescence in transfected cells was measured by analysing the images captured using the software ImageJ. For all applications, the images were first background subtracted using the ‘subtract background’ application which employs a rolling ball radius algorithm and considers intensity values outside the radius to be background.

For the GFP fluorescence quantification in chapter 3, the images were converted to grey scale and the mean grey value of each image was measured. The mean grey value is the mean of brightness value of all individual pixels within an image. Hence the mean grey value of an image is directly proportional to the GFP fluorescence intensity for that image.

2.11 Other computer software applications

Sequencing data was analysed using Chromas Lite 2.0 or Clone Manager, SciEd Central, v7.04. Plasmid maps detailing the position of the genes, the restriction enzyme sites and promoters were constructed by using Clone Manager, SciEd Central, v7.04 and PlasMapper version 2.0 software. Band densitometry of the proteins within western blots was carried out using Scion Image software. The bar charts presented in this study were prepared using GraphPad Prism 5. The statistical analyses of the numerical data in this study were carried out using GraphPad InStat 3.

CHAPTER 3

Mapping the site of interaction of Vif and

APOBEC3G with Pr55^{GAG}

3.1 Background:

The *vif* gene of HIV-1 is essential for HIV infectivity in Peripheral Blood Mononuclear Cells (PBMCs), the natural host cells for HIV. One of the functions of Vif is to antagonise the inhibition of virus replication imposed by APOBEC3G (A3G) which is constitutively expressed in PBMCs and a component of the human innate immune response.

Following the recognition that Vif is indispensable to HIV in overcoming the inhibition exerted by A3G, considerable attention has been focussed on the mechanism by which Vif executes this function and allows viral replication and infectious virus production in its normal host cells.

Initially a relatively simple picture emerged, whereby Vif expressed in the cytoplasm of HIV infected cells was found to form a Vif-Cul5-SCF complex that recruited A3G leading to its polyubiquitination and forwarding to the 26S Proteasome for degradation (Yu et al, 2003). However more recently there have been a number of conflicting reports emerging in the literature which have undermined this straightforward model.

One of the first studies to challenge the initial model of Vif action demonstrated that even though a low level of A3G was detected in Vif+ HIV-1 infected cells, the progeny virions still remained infectious suggesting that Vif was exerting some other as yet uncharacterised mechanism to exclude A3G from the budding progeny virions (Kao et al, 2004). Further evidence came in form of the report in which the block to virus replication of a degradation resistant mutant of A3G could still be alleviated by Vif (Opi et al, 2007). A second independent line of evidence established that the

fusion of A3G to a fragment of the viral Vpr protein allows it to overcome Vif's relief of the block even though it is still degraded by Vif (Ao et al, 2008). Consequently a view is emerging that whilst there has been rapid progress in trying to dissect Vif's role in overcoming the A3G based facet of the inherent immune response against HIV-1, a detailed knowledge about the mechanism involved is not yet clear.

3.2 New hypothesis for the primary role of Vif in overcoming A3G based inhibition of HIV replication:

Vif and A3G have both been shown to interact specifically with Pr55^{GAG} and become incorporated into the core of budding HIV virions (Bouyac et al, 1997a; Cen et al, 2004; Huvent et al, 1998; Schafer et al, 2004; Syed & McCrae, 2009). **The hypothesis for Vif's primary role in alleviating the A3G block that this study seeks to test is that Vif outcompetes A3G for Pr55^{GAG} binding, thereby preventing its incorporation into budding progeny virions and subsequent inhibitory effects on HIV replication.**

This explanation of Vif's primary role in circumventing this facet of the innate immune response would in its simplest form require both Vif and A3G to interact with the same site on Pr55^{GAG}. Consequently the first experimental objective was to establish if this was the case or not and the experiments described in this chapter try to address this question.

3.3 Experimental strategy

It was decided to insert mutations in the regions of interest within the Pr55^{GAG} sequence and study the effect of these mutations on the Vif-Pr55^{GAG} and A3G-Pr55^{GAG} interactions. The regions of interest within the Pr55^{GAG} sequence were defined on the basis of previous reports addressing these interactions (Burnett & Spearman, 2007; Luo et al, 2004). Most of those studies pointed towards the involvement of the Nucleocapsid (NC) domain of Pr55^{GAG} in interactions with both Vif and A3G (Burnett & Spearman, 2007; Luo et al, 2004).

To shed light on the interactions of both Vif and A3G with the NC domain, it is essential to first understand the sequence and the structure of the NC portion of Pr55^{GAG}. Fig. 3.1 (A), (B) & (C) depict the domain organisation of Pr55^{GAG}, the amino acid sequence of its NC domain and the 3-D structure of NC bound to the RNA stem-loop structure SL3 of the genomic Psi RNA packaging signal respectively. Residues K3-R10 at the N-terminal of the NC protein form a helix that binds to the RNA major groove of SL3 genomic Psi RNA {Fig. 3.1 (C)}. In addition there are two zinc finger knuckles (V13-A30 & G35-E51) which are separated by the basic linker segment (P31-K34) {Fig. 3.1(B)}. The zinc finger domains along with the basic linker are involved in the interactions with the exposed bases of the RNA tetraloop of the SL3 Psi genomic RNA packaging signal {Fig. 3.1 (C)}. Thus the N-terminal region of NC along with the two zinc fingers and basic linker are involved in the interaction and subsequent encapsidation of the viral genomic RNA into newly formed virus particles. The NC sequence ends with a C-terminal tail spanning amino acids R52 to N55 {Fig. 3.1 (C)} (De Guzman et al, 1998).

Little was known about the position at which Vif might interact within the NC domain of Pr55^{GAG} (Huvent et al, 1998). On the other hand, the A3G-Pr55^{GAG} interaction was slightly better characterised. A previous study had reported that the 'T' domain spanning residues M1-K11 at the N-terminal of the NC portion of Pr55^{GAG} was indispensable for the A3G-Pr55^{GAG} interaction {Fig. 3.1 (B)} (Luo et al, 2004). Hence the mutant NCDel was created by the deletion of amino acids G4 – T12 from the N-terminal of NC leaving the first three amino acids which are a part of the viral protease cleavage site intact {Fig 3.1 (B) & (C)}. A more recent report however suggested that it was not the 'T' domain but the basic linker segment present between the two zinc finger motifs of the NC which was involved in the interaction of A3G with the polyprotein GAG precursor {Fig. 3.1 (B)} (Burnett & Spearman, 2007). Consequently a substitution mutant NCSub was also constructed by substituting amino acids 32RKK34 within the basic linker with 32EEK34 thereby inserting two acidic amino acids (E-Glutamic acids) in place of two basic ones (32R-Arginine and 33K-Lysine) {Fig 3.1 (B) & (C)}.

Both of these mutants NCDel and NCSub were tested for interactions with Vif and A3G to find out whether these two proteins interacted at the same site on Pr55^{GAG}.

3.4 Assay system:

The primary assay used to study interactions between Vif/A3G and Pr55^{GAG} was the mammalian two-hybrid assay.

3.4.1 Introduction to the two-hybrid assay:

The two-hybrid assay system is an assay protocol used to study protein-protein interactions *in vivo*. It was first developed using the GAL4 transcription factor of the

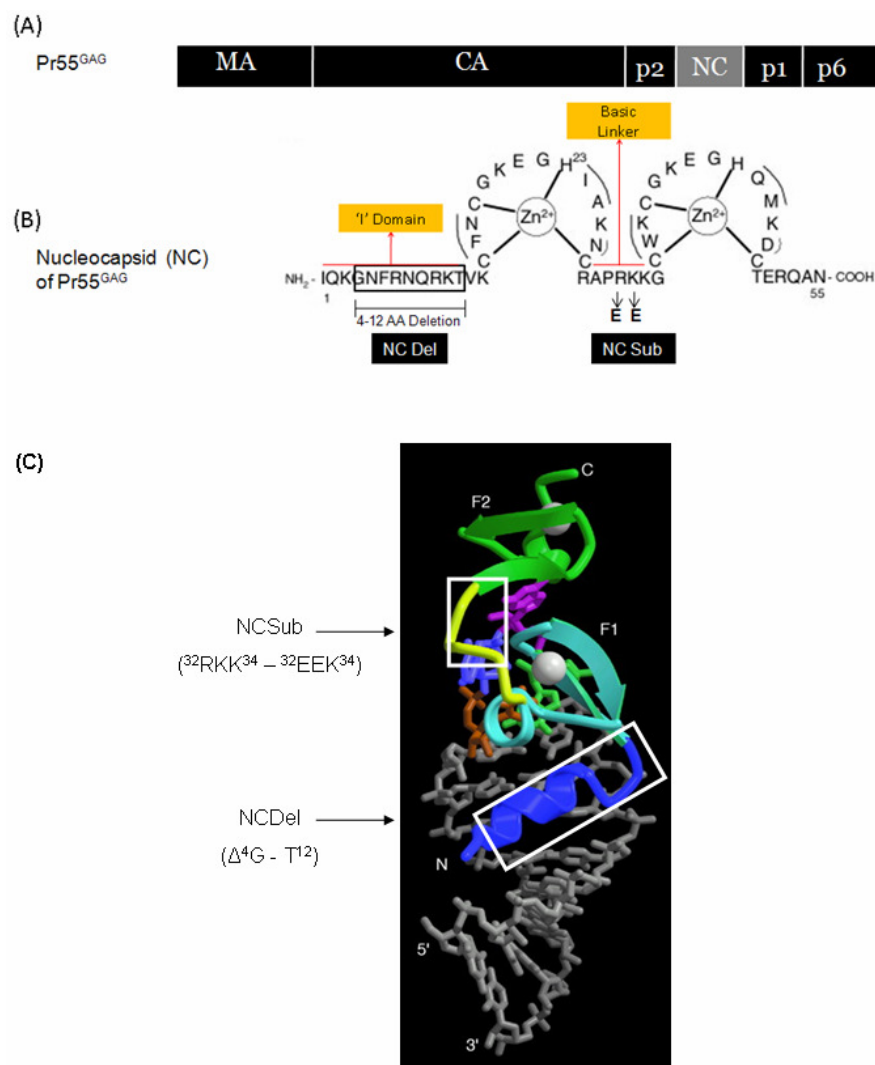


Figure 3.1 Diagrammatic representation of the position of the mutations within Pr55^{GAG}. {A} Domain organisation of Pr55^{GAG} with the Nucleocapsid (NC) region highlighted (grey). {B} NC region of Pr55^{GAG}: (i) NCDel: Deletion of amino acid 4-12 region within the NC sequence (ii) NCSUB: Substitution of two basic amino acids, R (Arginine) and K (Lysine) with two acidic amino acids, E (Glutamic acid). {C} Ribbon diagram of the HIV-1 NC and SL3 Ψ RNA complex with the position of NCDel and NCSUB mutations highlighted. Colour code: N-terminal helix (dark blue), 1st Zinc finger F1 (sky blue), basic linker (yellow), 2nd Zinc finger F2 (green) and RNA (grey, except for the bases in contact with the zinc fingers and basic linker which are coloured). The white beads represent the zinc atoms bound to the two zinc fingers. The positions of the mutants NCDel and NCSUB are highlighted with white rectangles. Figure modified from De Guzman et al, 1998.

yeast *Saccharomyces cerevisiae* (Fields & Song, 1989).

It is based on the principle that many eukaryotic transcription factors have two distinct physically separable functional domains. In the case of the GAL4 transcription factor these are a GAL4 DNA binding domain which binds to the Upstream Activation Sequence (UAS) and an activation domain which initiates gene transcription. Normally these two domains form an integral part of the GAL4 protein and function as a single unit. However, in the two-hybrid assay, the two domains are physically separated and fused in frame with two genes of interest thought to interact with each other. If the two proteins do indeed form a protein-protein complex and hence bring the two GAL4 domains near each other, then their proximity is sufficient to reconstitute a functional GAL4 transcription factor and initiate transcription of any gene lying downstream of the GAL4 responsive UAS (Fig. 3.2).

The yeast two-hybrid assay has a number of advantages over other assay protocols for investigating protein-protein interactions. First, it does not require purification of the candidate proteins to be studied, nor does it require any antibodies to these proteins to be available. Secondly, because the proteins being analysed are expressed *in vivo*, they are more likely to retain their native conformation. The assay has also been found to have a good dynamic range and hence useful for the detection of interactions occurring transiently/weakly within cells. All these merits make it a very useful technique for the study of protein-protein interactions *in vivo*.

A further advantage of the two-hybrid assay is, having established that an interaction between two proteins exists, then it is an extremely potent tool for mapping the regions of the interaction through the use of deletion/site-directed mutagenesis of the entities involved.

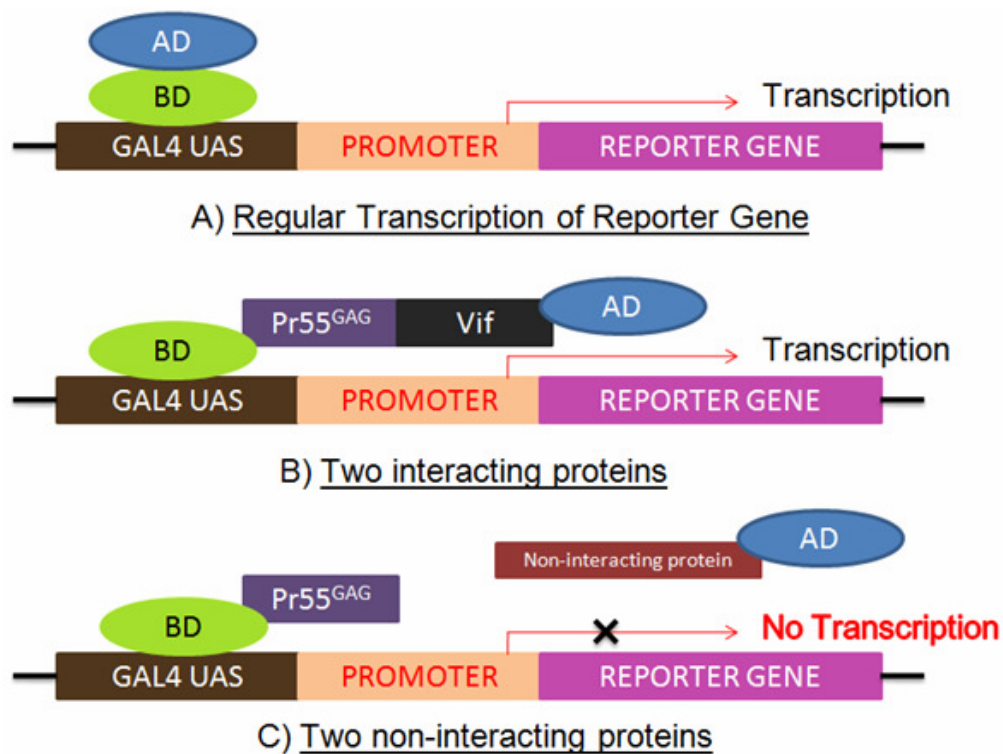


Figure 3.2 Working Principle of the mammalian two-hybrid assay: {A} Regular transcription of the reporter gene under the control of a GAL4 responsive promoter when the Activation domain (AD) and DNA binding domain (BD) are intact. {B} In the two-hybrid system, the two domains are physically separated and fused to the two genes of interest (here, Pr55^{GAG} and Vif). As Vif and Pr55^{GAG} interact with each other, the two domains come into proximity to each other initiating reporter gene transcription. {C} In the case of two non-interacting proteins, the two domains remain separated and there is no reporter gene transcription

Finally an important application of the two-hybrid assay system has been its use in screening cDNA libraries for cellular interacting partners of a protein of interest (Chien et al, 1991). This application has been used widely to detect and decipher cellular interaction networks. The ease with which the cloned cDNAs can be isolated to yield positive interaction partners makes it one of the methods of choice for screening cDNA libraries.

3.4.2 The mammalian two-hybrid system:

The mammalian two-hybrid assay is a modification of the yeast two-hybrid protocol. It incorporates all the advantages of the yeast two-hybrid system and has the additional benefit of the proteins being expressed in mammalian cells, rather than yeast cells. This allows the method to be used to detect protein interactions which are dependent on mammalian type post-translational modification of the proteins involved, which is not possible with the yeast two-hybrid assay.

3.4.3 Two-hybrid assay applications in HIV research

The yeast two-hybrid assay has already been used to detect protein-protein interactions between different Human Immunodeficiency Virus proteins. One of the earliest application dates back to 1992, when Luban *et al* used the two-hybrid assay to demonstrate multimerization of retroviral Gag polyproteins (Luban et al, 1992). Later, it was employed to show a direct interaction between the viral accessory protein Vpr and the Matrix protein of HIV-1 (Sato et al, 1996). It has also been exploited to screen cDNA libraries for elucidating virus-host cell protein interactions (Lama & Trono, 1998).

3.5 Vectors used in the Mammalian two-hybrid assay:

3.5.1 Two-hybrid assay vectors

Two expression plasmids are employed in the mammalian two-hybrid assay; pM (DNA binding domain, BD) cloning vector is used to construct a fusion gene encoding the chimeric protein of interest linked to the GAL4 DNA binding domain. Similarly, pVP16 (Activation domain, AD) cloning vector is used to construct a fusion gene encoding second chimeric fusion protein containing the transcriptional activation domain derived from the VP16 protein of the Herpes Simplex Virus (Fig. 3.3). These two plasmids are available commercially as part of Clontech's Matchmaker TM assay system and were used to generate additional constructs to those available from previous work done at Warwick. These plasmids AD-Vif, AD-A3G and BD-Pr55^{GAG} encoding the respective proteins fused in frame with either the Activation Domain (AD) or the DNA Binding Domain (BD) under the control of the SV40 promoter have been described previously (Syed & McCrae, 2009). The plasmid BD-A3G has previously been shown to be self-activating within the two-hybrid system and hence was not used in the experiments described in this study (Syed, 2008).

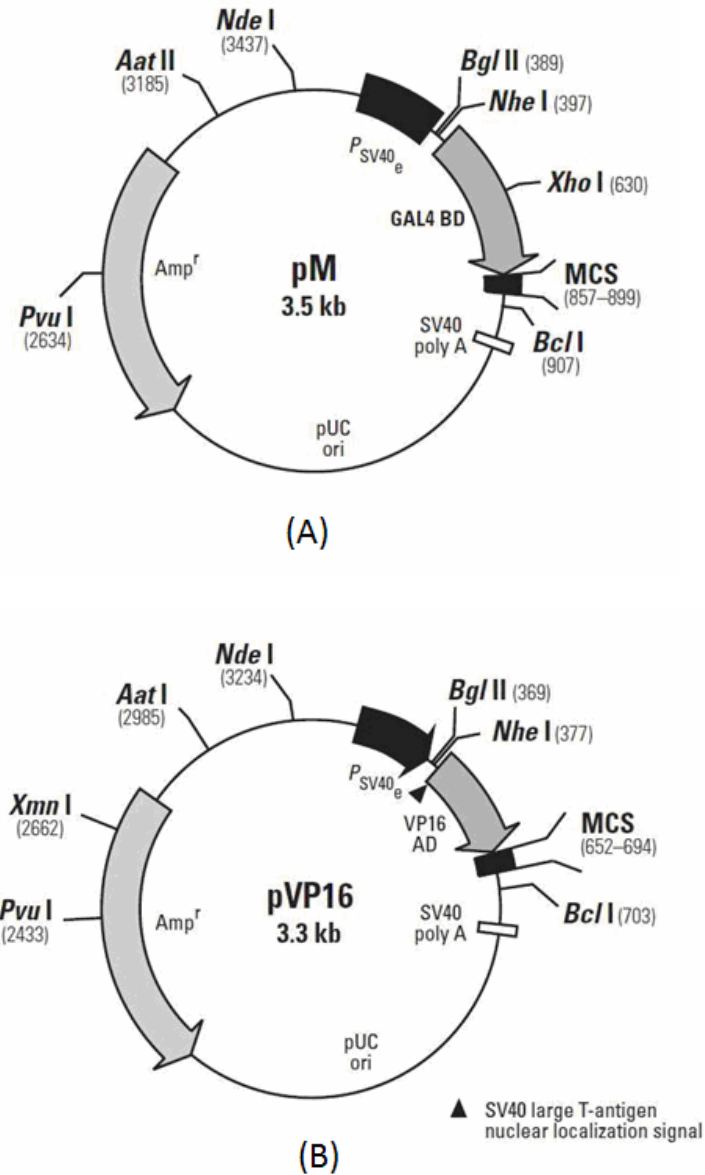


Figure 3.3 Mammalian two-hybrid assay vectors: {A} pM Vector: The pM vector is used to generate a fusion protein of the GAL 4 DNA Binding domain (BD) with the protein of interest. There is a nuclear localisation signal within the GAL4 sequence which directs the chimeric protein to the nucleus. {B} pVP16 Vector: The pVP16 vector is used to generate a fusion protein of the VP16 Activation domain (AD) with the protein of interest. There is the SV40 nuclear localisation signal within the VP16 AD sequence which targets the fusion protein to the nucleus. Transcription is initiated from the SV40 promoter in both the vectors. Genes of interest are cloned using the multiple cloning sites such that the genes are in frame with the BD and AD respectively in both the vectors.

3.5.2 Generation of Pr55^{GAG} mutants

Two Pr55^{GAG} mutants were used in the assay. BD-NCDel was constructed by first inserting a HpaI restriction enzyme site into the coding sequence of Pr55^{GAG} by site-directed mutagenesis of the BD- Pr55^{GAG} plasmid using a PCR based mutagenesis protocol with the forward primer GagHpaF and the reverse primer GagHpaR (Table 2.2.1) to generate BD-Pr55^{GAG}-HpaI (Fig. 3.4B). A further PCR based mutagenesis of the BD-Pr55^{GAG}-HpaI using the forward primer EcoGagF and the reverse primer HpaIGagR was then carried out to delete amino acids 4-12 from the amino terminus of the NC region of Pr55^{GAG} (Table 2.2.1)(Fig. 3.4C). The PCR product and BD-Pr55^{GAG}-HpaI vector were digested with EcoRI and HpaI so that the insert could be ligated into the parent vector resulting in a deletion of 4-12 amino acids from the N-terminal of NC domain of Pr55^{GAG} (BD-NCDel) (Fig. 3.1 , 3.4 D & Fig. 1.1Appendix). A second Pr55^{GAG} mutant, BD-NCSUB was constructed by site-directed mutagenesis of BD-Pr55^{GAG} again using PCR based mutagenesis using the forward primer 1551GagMutF and the reverse primer 1551GagMutR (Table 2.2.1). This produced a mutated Pr55^{GAG} in which there had been the substitution of two basic amino acids (Arginine and Lysine) with two acidic ones (Glutamic acid) in the basic linker of the NC region of the protein (BD-NCSUB) {Fig. 3.1 and Fig. 1.2 Appendix}.

3.5.3 Reporter vectors:

Two reporter plasmids pUAST-hrGFP-neo and pG5CAT (Clontech) were employed in the two-hybrid assay containing respectively the Green Fluorescent protein (GFP) gene and Chloramphenicol Acetyl Transferase (CAT) gene downstream of the GAL4

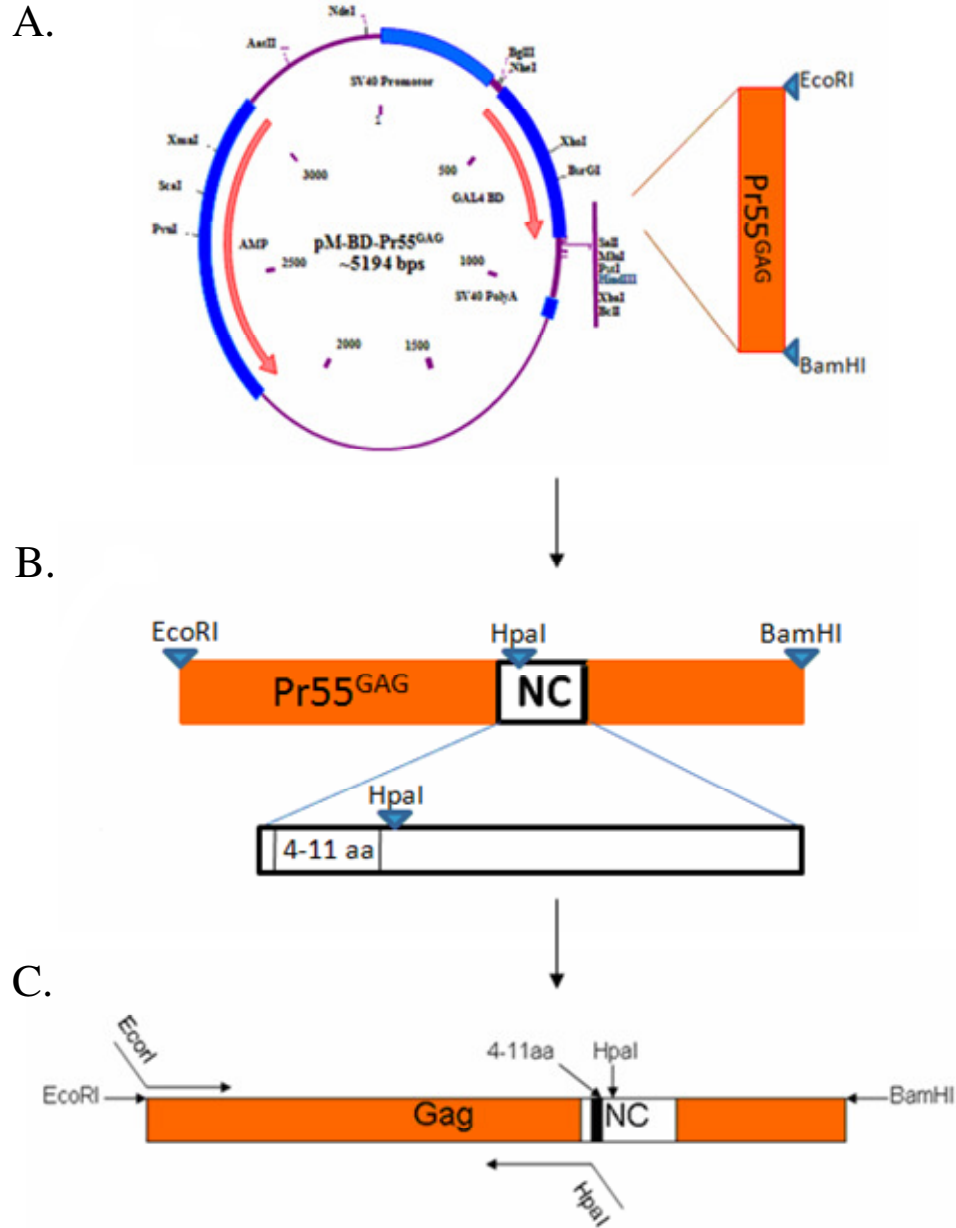


Figure 3.4 Schematic of construction of the Pr55^{GAG} mutant BD-NCDel. {A} BD-Pr55^{GAG}: Schematic diagram showing Pr55^{GAG} flanked by restriction enzymes EcoRI and BamHI and inserted in frame downstream of the DNA binding domain of pM vector. {B} Construction of BD-Pr55^{GAG}-HpaI: Insertion of an HpaI restriction enzyme site immediately downstream of the first 11 amino acids at the N-terminus of the Nucleocapsid (NC) domain of Pr55^{GAG} by PCR based site-directed mutagenesis of BD-Pr55^{GAG}. {C} PCR based mutagenesis of BD-Pr55^{GAG}-HpaI to delete amino acids 4-12 near the amino terminus of the NC region of Pr55^{GAG}.

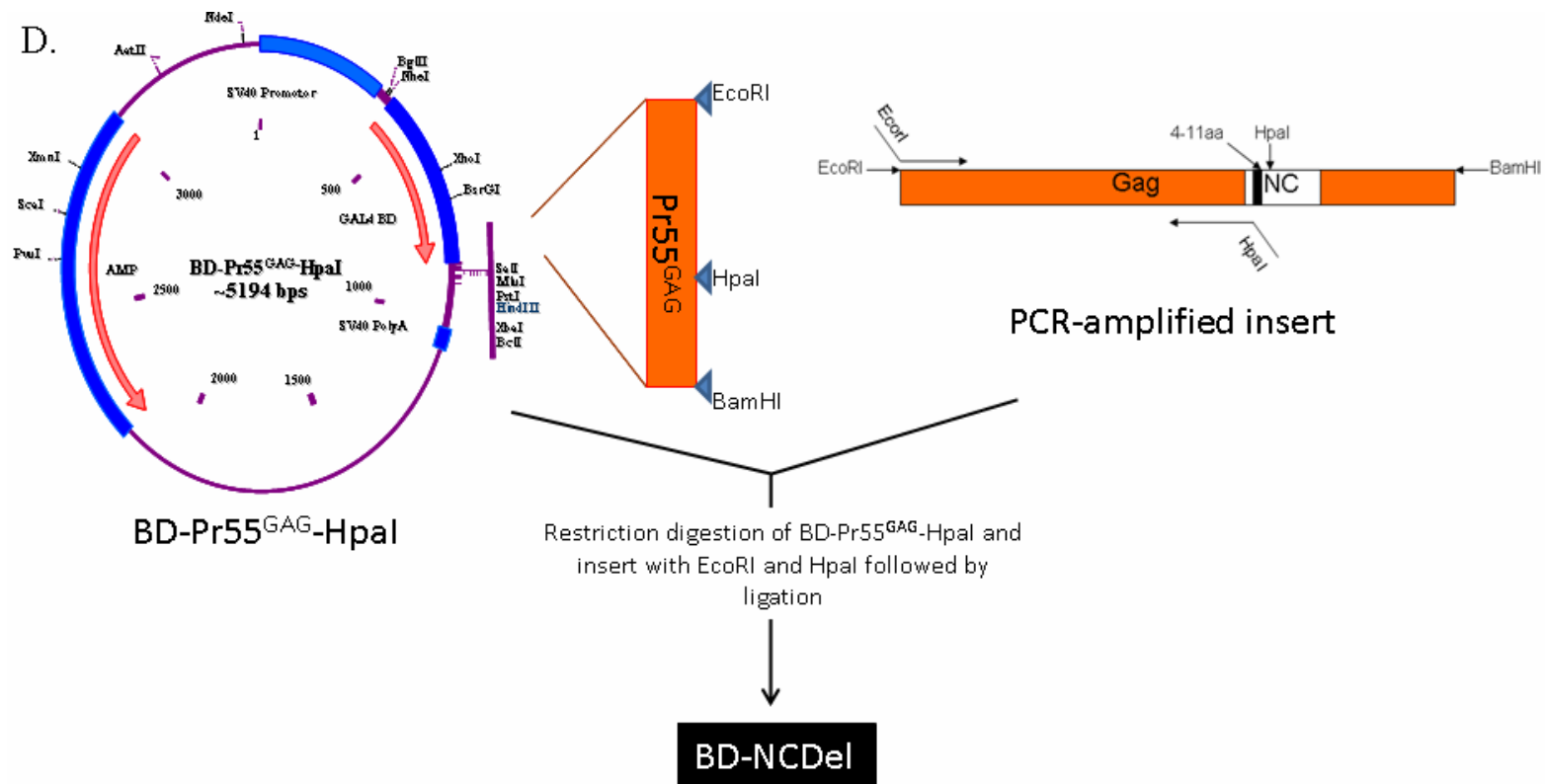


Figure 3.4 Schematic of construction of the Pr55^{GAG} mutant BD-NCDeI (Contd.) {D} Restriction digest of both BD-Pr55^{GAG}-HpaI and the PCR amplified insert shown in C with enzymes EcoRI and HpaI. Digested vector and insert were ligated to form BD-NCDeI, a mutant version of Pr55^{GAG} where amino acids 4-12 from the amino terminus of the Nucleocapsid are deleted from the Pr55^{GAG} coding sequence.

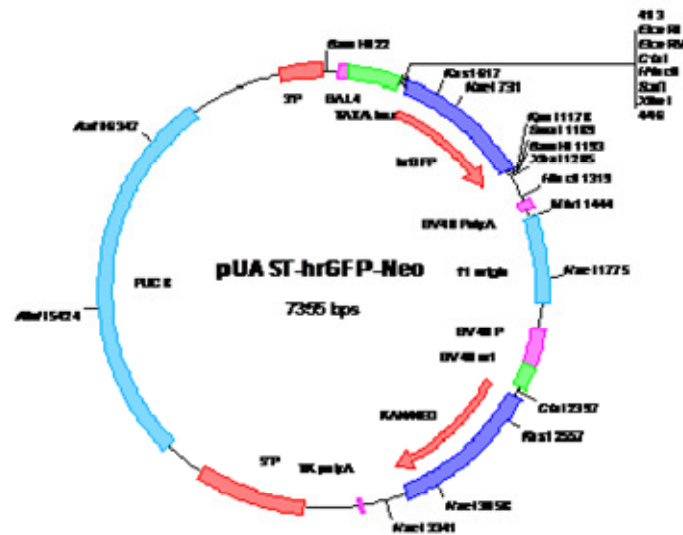
responsive element and the minimal promoter of the Adenovirus E1b gene (Fig. 3.5). GFP was chosen for use as an initial qualitative reporter (pUAST-hrGFP-neo) because its expression could be easily and rapidly screened in live cells. The GFP expression level within cells was later analysed with ImageJ image analysis software. The GFP expression data was then confirmed using the quantitative CAT reporter (pG5CAT) by employing the CAT ELISA to gauge the change observed in strength of any interactions seen.

3.5.4 Assay controls:

The positive controls used throughout this study exploited the previously reported interaction between murine p53 and SV40 Large-T antigen (Ali & DeCaprio, 2001). The plasmid pM-p53 which encodes a chimeric murine protein p53 fused in frame with the DNA Binding Domain of the pM vector was used together with the pVP16-T plasmid which encodes the chimeric SV-40 Large-T antigen fused to the Activation Domain of the pVP16 vector. When pM-p53 and pVP16-T were co-transfected with either reporter plasmid, positive reporter gene expression was seen.

The negative controls used for the two-hybrid assays were individual plasmids expressing the AD or BD fusion protein constructs of interest together with either empty BD or empty AD vector respectively. The following combinations were employed: (a) AD-A3G + empty BD vector (b) AD-Vif + empty BD vector (c) BD-Pr55^{GAG} + Empty AD vector (d) BD-NCDel + Empty AD vector and (e) BD-NCSUB + Empty AD vector. Cells were transfected separately with all these various combinations of plasmids together with either GFP or CAT reporter vector to monitor the background levels of reporter gene expression and rule out the non-specific self-activation of the reporter vectors with single plasmids.

A.



B.

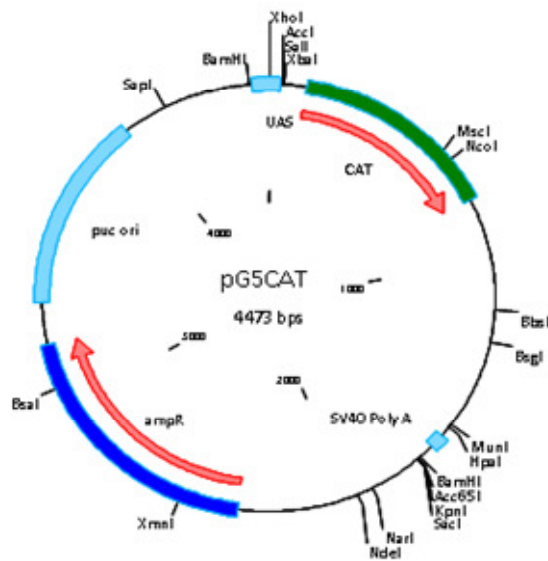


Figure 3.5 Reporter vectors used in the mammalian two-hybrid assay. {A} pUAST-hrGFP-Neo: Contains the Green Fluorescent Protein (GFP) gene positioned downstream of the GAL4 responsive element and a minimal promoter from the Adenovirus E1b gene. It is used as a qualitative reporter in the two-hybrid assay as the GFP expression can be easily observed under the UV microscope in live cells. {B} pG5CAT: Contains the Chloramphenicol Acetyl Transferase (CAT) gene positioned downstream of the GAL4 responsive element and a minimal promoter from the Adenovirus E1b gene. It is used as a quantitative reporter to both confirm the qualitative reporter data whilst at the same time allowing an estimation to be made about the relative strength of the interactions.

3.6 Assay protocol

The mammalian two-hybrid assay protocol has been described in detail in section 2.7 of the Materials and Methods. Each well was transfected with 1.5 µg of each of the plasmids used. The cells were analysed as described in section 2.7.

3.7 Mapping the regions of interaction on Pr55^{GAG} where Vif and A3G interact:

Some studies aimed at mapping the region of Pr55^{GAG} that interacts with A3G have already been reported before in this chapter (Burnett & Spearman, 2007; Luo et al, 2004). The initial work mapped site of interaction between these two proteins to the so called I domain located at the amino terminus of the Nucleocapsid (NC) portion of Pr55^{GAG} (Fig. 3.1 B) (Luo et al, 2004). However a subsequent report localised the interaction site to the basic linker region of NC which lies further into the protein between two zinc finger domains (Fig. 3.1 B) (Burnett & Spearman, 2007). To investigate the importance of both these regions on the interaction of A3G and/or Vif with Pr55^{GAG}, the mutants NCDel and NCSub described in sections 3.3 and 3.5.2 were used (Fig. 3.1 B and C).

The interactions were first studied by using the GFP reporter vector and monitoring the level of GFP expression to estimate the presence of an interaction where a high level of GFP expression indicated that the interaction was present while background level GFP expression indicated no interaction (Fig. 3.6, 3.8 and 3.11). When COS-1 cells transfected with BD-p53 and AD-LargeT along with GFP reporter vector were observed under UV light microscope, many cells were positive for GFP expression indicating that the two proteins interacted with each other in this system. This protein

pair was the positive control for the assay (Fig. 3.6 A). The negative controls for the assay were single plasmids expressing chimeric proteins of interest fused to either the BD/AD domains transfected in cells along with empty AD/BD vectors respectively and GFP reporter vector. Thus cells transfected with AD-Vif and empty BD vector along with GFP reporter gave background level GFP expression indicating that AD-Vif does not self-activate the GFP reporter (Fig. 3.6 B). Similarly cells transfected with AD-A3G and empty BD, BD-Pr55^{GAG} and empty AD, BD-NCDel and empty AD & BD-NCSub and empty AD also gave very low GFP reporter expression pointing that these plasmids on their own did not activate reporter expression (Fig. 3.6 C, D, E and F).

Transfection of the same positive and negative controls was then carried out by employing the CAT reporter vector instead of GFP. CAT enzyme values were then measured by following the standard CAT ELISA protocol on extracts of transfected cells as mentioned in section 2.7. As expected, in case of the CAT reporter vector, the positive control yielded high CAT enzyme values while the negative controls mentioned above resulted in background level CAT enzyme expression (Fig. 3.9 A and 3.12 A).

After ascertaining that the positive and negative controls for the assay were functioning optimally with both the GFP and CAT reporter vectors, the next step was to study the interactions of both Vif and A3G with Pr55^{GAG} and the two GAG mutants NCDel & NCSub.

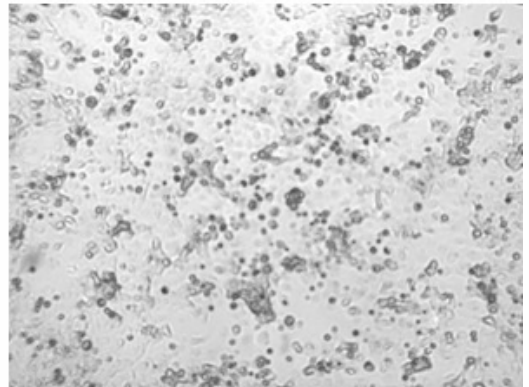
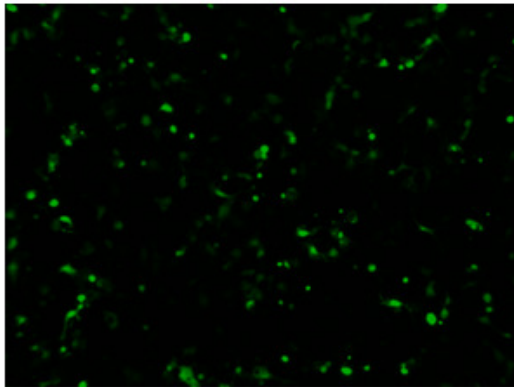
Figure 3.6 GFP reporter expression levels of the positive and negative controls for the mammalian two-hybrid assay: COS-1 cells were transfected with the respective plasmids using a standard Lipofectamine transfection protocol detailed in section 2.5.3. Live cells were observed for GFP expression 48 hours post transfection under UV light microscope (section 2.7). The positive control for the assay exploited the previously reported interaction between murine p53 and SV40 large T antigen. The negative controls for the assay were individual plasmids expressing AD/BD domain fused chimeric proteins of interest together with either empty BD or empty AD vector respectively. In addition to the plasmid combinations mentioned below, cells were also co-transfected with the GFP reporter vector whose expression was under the control of a GAL4 responsive element. Monitoring the levels of GFP reporter gene expression enabled the readout of whether an interaction was present between two proteins of interest or not. Panels on the right hand side show visible light image of the transfected cells and panels on the left display fluorescence of GFP under UV light.

The following plasmid combinations were employed:

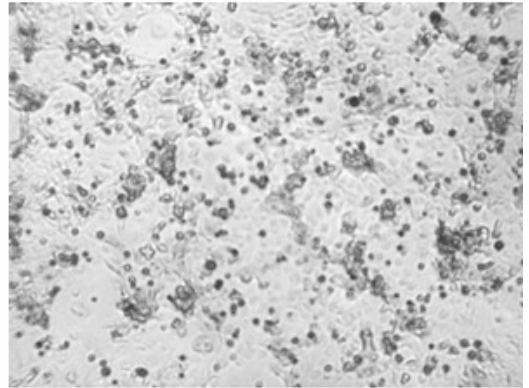
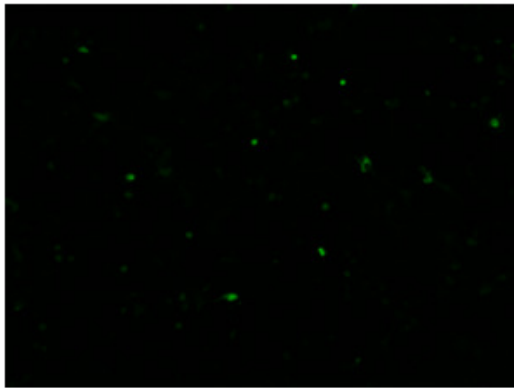
- {A} Positive control: BD-p53 + AD-LargeT along with GFP reporter vector
- {B} Negative control 1: AD-Vif + Empty BD vector along with GFP reporter vector
- {C} Negative control 2: AD-A3G + Empty BD vector along with GFP reporter vector

(A)

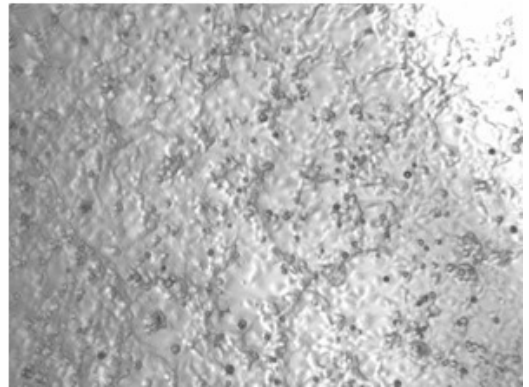
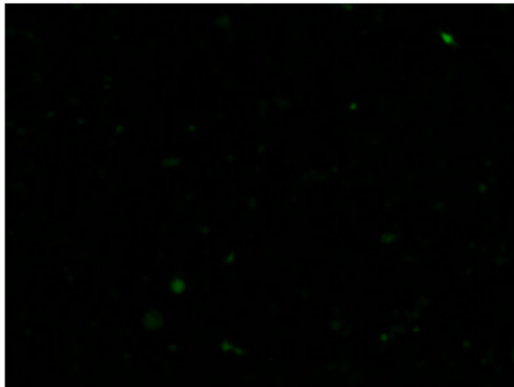
40X Magnification



(B)



(C)



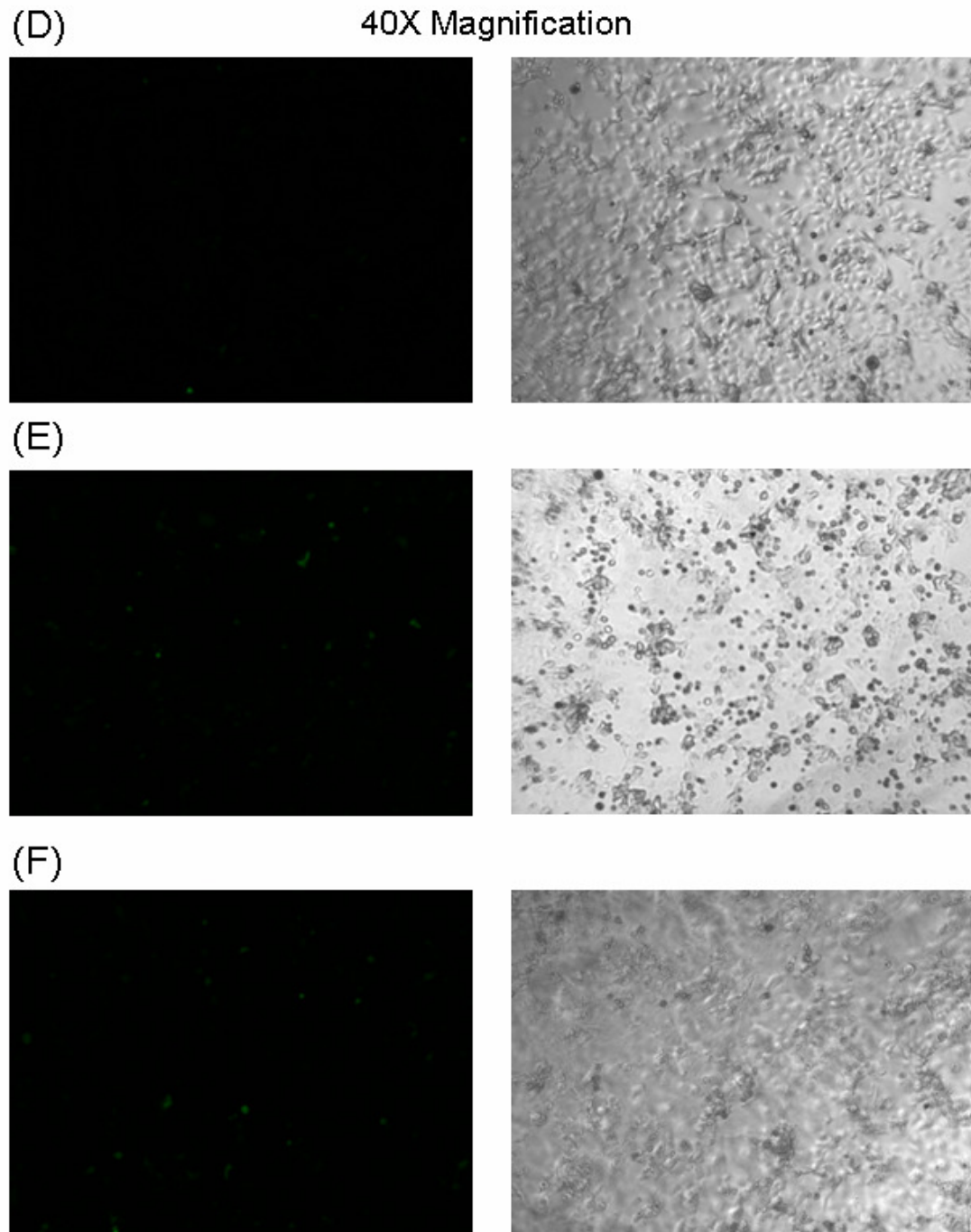


Figure 3.6 (contd.) GFP reporter expression levels of the positive and negative controls for the mammalian two-hybrid assay

{D} Negative control 3: BD-Pr55^{GAG} + Empty AD vector with GFP reporter vector

{E} Negative control 4: BD-NCDe1 + Empty AD vector with GFP reporter vector

{F} Negative control 5: BD-NCSuB + Empty AD vector with GFP reporter vector

3.7.1 A3G-Pr55^{GAG} interaction

When cells were transfected with AD-A3G and BD-Pr55^{GAG} along with GFP reporter vector, positive GFP expression was observed indicating that A3G and Pr55^{GAG} specifically interact with each other (Fig. 3.7 A). Similarly co-transfection with AD-A3G and BD-NCDel along with GFP reporter resulted in positive GFP expression (Fig. 3.7B). This result was unexpected as the deleted region in NCDel had been shown in a previous report to mediate the interaction between A3G and Pr55^{GAG} (Luo et al, 2004). Thus this result did not support Luo *et al.*'s conclusions that the N-terminal 'I' domain of NC was important in mediating the A3G-Pr55^{GAG} interaction. By contrast, when cells were co-transfected with AD-A3G and BD-NCSUB along with GFP reporter, a huge drop in GFP expression was observed and the GFP expression almost became comparable to negative control levels (Fig. 3.7C). This result was in support of Burnett and Spearman's conclusions that the basic linker region in the NC domain of Pr55^{GAG} was important in mediating the interaction of A3G with Pr55^{GAG}.

Later, the images were analysed using ImageJ software for the intensity of GFP expression by converting the RGB images to gray scale and measuring the mean gray value for each image as described in section 2.10 (Fig. 3.8 A). The mean gray value of the image was directly proportional to the intensity of GFP expression.

When a bar chart was plotted to show the relative GFP intensity using the mean gray values in Fig 3.8 A, the GFP intensity for AD-A3G and BD-Pr55^{GAG} was almost 2.5 times more than that of the positive control (Fig. 3.8 B). The reason for the relatively lower values of reporter gene expression observed with the positive control could be due to the cell line employed for these studies. COS-1 cells used in this assay carry

an integrated copy of the SV40 genome and constitutively express biochemically active SV-40 encoded LargeT antigen. The positive control for this experiment was the interaction between BD-p53 and AD-LargeT. Hence the endogenously expressed LargeT protein in COS-1 cells could be competing with the chimeric AD-fused LargeT protein used in the two-hybrid assay for binding with BD-p53. This sequestration of BD-p53 by the endogenously expressed LargeT was possibly being reflected in form of reduced levels of reporter gene expression.

The GFP intensity with AD-A3G and BD-NCDel was even more when compared with AD-A3G and BD-Pr55^{GAG} but this increase was statistically not significant ($p>0.05$) (Fig. 3.8 B). In contrast, the GFP intensity of AD-A3G and BD-NCSub was significantly reduced ($p<0.001$) when compared with AD-A3G and BD-Pr55^{GAG} and was almost comparable to negative control levels (Fig. 3.8 B). This significant drop in GFP intensity observed with NCSub indicated that the substitution mutations in the basic linker of NC had a deleterious effect on the A3G-Pr55^{GAG} interaction.

Thus from the GFP expression data it was clear that the mutations in the NCSub mutant of Pr55^{GAG} were responsible for disrupting the A3G-Pr55^{GAG} interaction. Contrary to a previous report, the mutations in NCDel mutant of Pr55^{GAG} had no effects on the A3G-Pr55^{GAG} interaction.

Figure 3.7 GFP reporter expression levels of the interactions between wild type Pr55^{GAG} and the two GAG mutants with A3G: COS-1 cells were transfected with the respective plasmids using a standard Lipofectamine transfection protocol detailed in section 2.5.3. Live cells were observed for GFP expression 48 hours post transfection under UV light microscope (section 2.7).

{A} BD-Pr55^{GAG} + AD-A3G: Wild type Pr55^{GAG} and A3G together with GFP reporter

{B} BD-NCDel + AD-A3G: NCDel mutant of Pr55^{GAG} and A3G together with GFP reporter

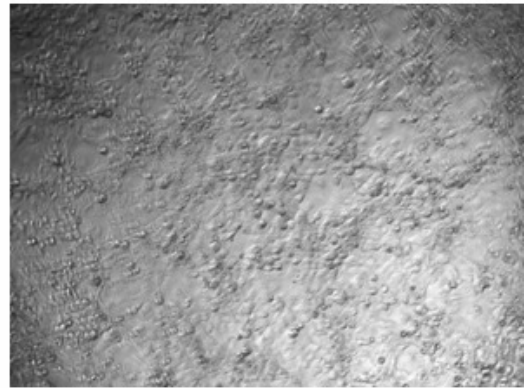
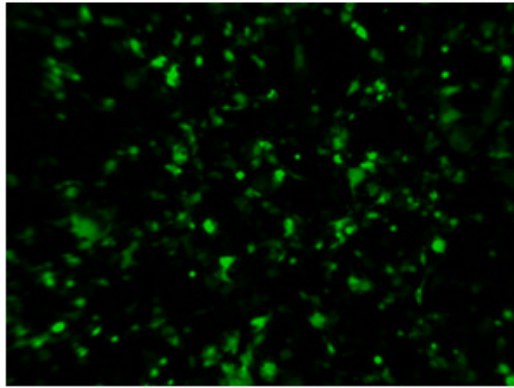
{C} BD-NCSub + AD-A3G: NCSub mutant of Pr55^{GAG} and A3G together with GFP reporter

The levels of GFP reporter expression were monitored to determine whether an interaction was present between the two proteins of interest or not whereby positive GFP expression indicated the presence of an interaction while no or background level GFP expression indicated no interaction.

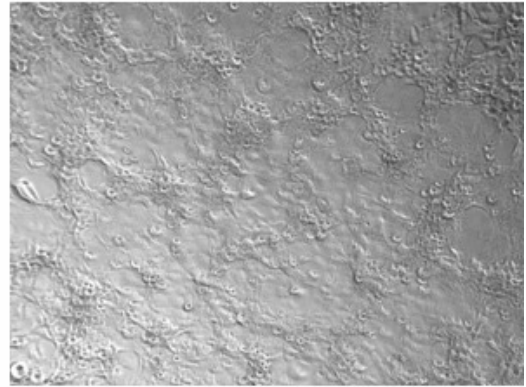
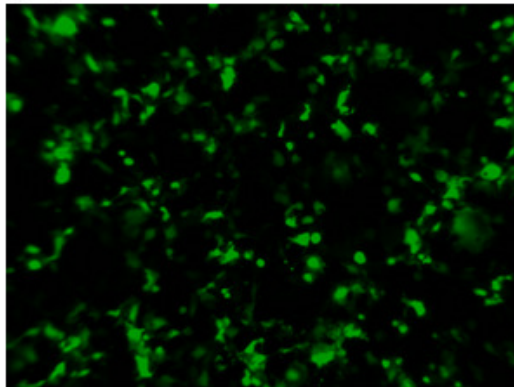
Panels on the right hand side show visible light image of the transfected cells and panels on the left display fluorescence of GFP under UV light.

(A)

40X Magnification



(B)



(C)

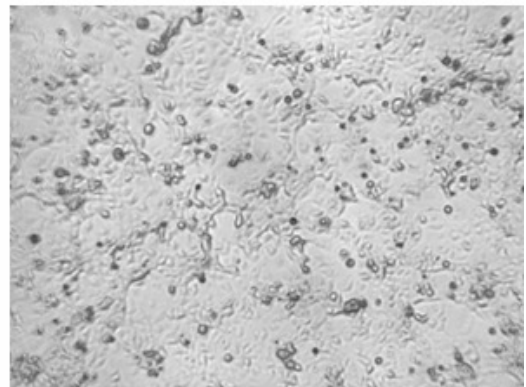
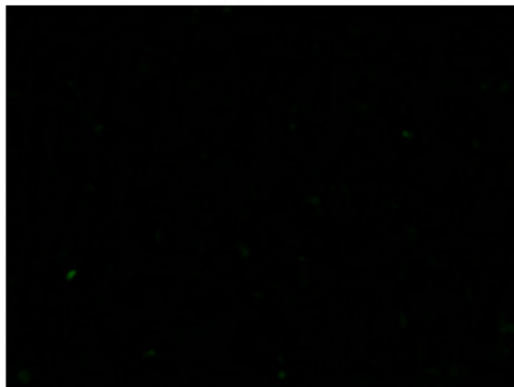


Figure 3.8 Quantifying the level of GFP expression using the image analysis software ImageJ to provide some indication of the strength of the interactions of A3G with wild type Pr55^{GAG} and the two GAG mutants: COS-1 cells were transfected with the indicated plasmid combinations in addition to the GFP reporter vector. 48 hours post-transfection, the cells were observed under UV light microscope and images were analysed for GFP expression using the image analysis software ImageJ. The RGB images were converted to gray scale to allow measurement of the gray values of each pixel within an image. The mean gray value of all pixels was thus directly proportional to the intensity of GFP.

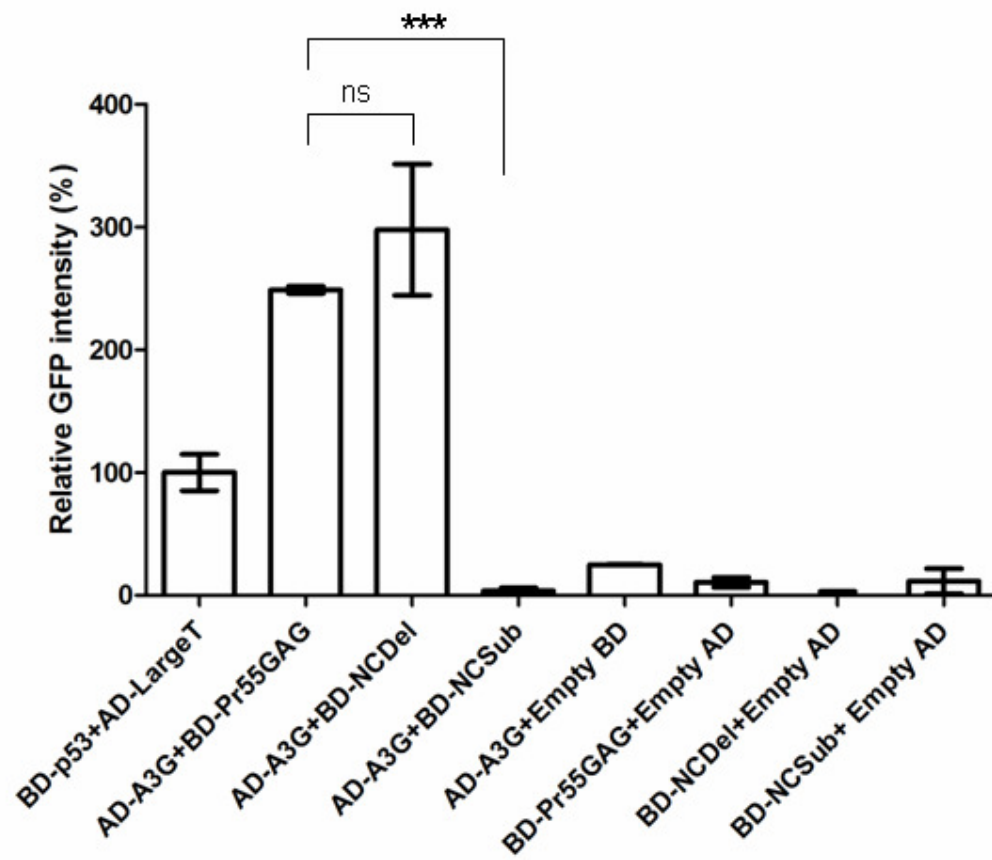
{A} Table depicting the mean gray values of images analysed using ImageJ against the plasmid combinations used in a transfection. The positive control for the assay exploited the interaction between murine p53 and SV40 Large T antigen. The negative controls for the assay were individual plasmids expressing AD/BD domain fused chimeric proteins of interest together with either empty BD or empty AD vector respectively.

{B} Bar chart depicting the GFP intensity within an image relative to the positive and negative controls (the positive control being 100% and the lowest negative control being 0%) drawn using the mean gray values in the table 3.8 {A}. Error bars indicate Mean \pm SEM (n=2 experiments). ns= difference not significant ($p>0.05$); ***= significant difference ($p<0.001$). The p values were calculated using one way ANOVA with post test.

(A)

Sr. No.	Plasmid combinations	Mean gray value (GFP Intensity/pixel)	
		Expt 1	Expt 2
1.	BD-p53 + AD-LargeT (Positive Control)	2.123	1.627
2.	AD-A3G +BD-Pr55 ^{GAG}	4.397	4.305
3.	AD-A3G + BD-NCDel	6.055	4.278
4.	AD-A3G + BD-NCSub	0.312	0.224
5.	AD-A3G + Empty BD (Negative control)	0.620	0.628
6.	BD-Pr55 ^{GAG} + Empty AD (Negative control)	0.452	0.320
7.	BD-NCDel + Empty AD (Negative control)	0.263	0.158
8.	BD-NCSub + Empty AD (Negative control)	0.571	0.232

(B)



To confirm the results obtained with the GFP reporter vector, the same experiments were repeated with CAT reporter vector and the results analysed by CAT ELISA on transfected cell extracts. Fig 3.9 A tabulates the absolute CAT values obtained against the various plasmid combinations employed for studying the A3G-Pr55^{GAG} interaction. BD-p53 and AD-LargeT along with CAT reporter vector gave positive CAT enzyme expression (positive control) (Fig. 3.9 A). Similar to what was observed with the GFP reporter vector, the CAT enzyme expression values obtained from cells transfected with AD-A3G and BD-Pr55^{GAG} along with CAT reporter vector were almost 1.75 times compared to those obtained from the positive control (Fig. 3.9 B).

Similarly when cells were transfected with AD-A3G and BD-NCDeI, the CAT enzyme expression was even more than that observed with AD-A3G and BD-Pr55^{GAG} (Fig. 3.9 B). This increase in CAT expression observed with NCDeI when compared to Pr55^{GAG} was statistically significant ($p < 0.01$) (Fig. 3.9 B). In case of the GFP reporter data, there was an increase in GFP intensity observed with the NCDeI mutant but this increase was found to be not significant ($p > 0.05$) (Fig. 3.8 B). Thus, in this case, there was an apparent discrepancy in the results obtained from the GFP reporter and CAT reporter data, the reasons for which have been discussed in the next section.

In contrast, when cells were transfected with AD-A3G and BD-NCSUB along with CAT reporter vector, there was a statistically significant decrease in the CAT enzyme expression when compared to that observed with AD-A3G and BD-Pr55^{GAG} ($p < 0.01$) (Fig. 3.9 B). This result was in agreement with the GFP reporter data mentioned earlier. Thus the basic residues (Arginine and Lysine) in the basic linker of the NC region of Pr55^{GAG} play an important role in mediating the A3G-Pr55^{GAG} interaction.

Figure 3.9 CAT reporter expression levels of the interactions between wild type Pr55^{GAG} and the two GAG Mutants with A3G: COS-1 cells were transfected with the respective plasmids using the standard Lipofectamine transfection protocol described in section 2.5.3. Cell extracts were analysed for CAT enzyme expression 72 hours post-transfection using a CAT ELISA kit (Roche) according to the manufacturer's instructions (section 2.7).

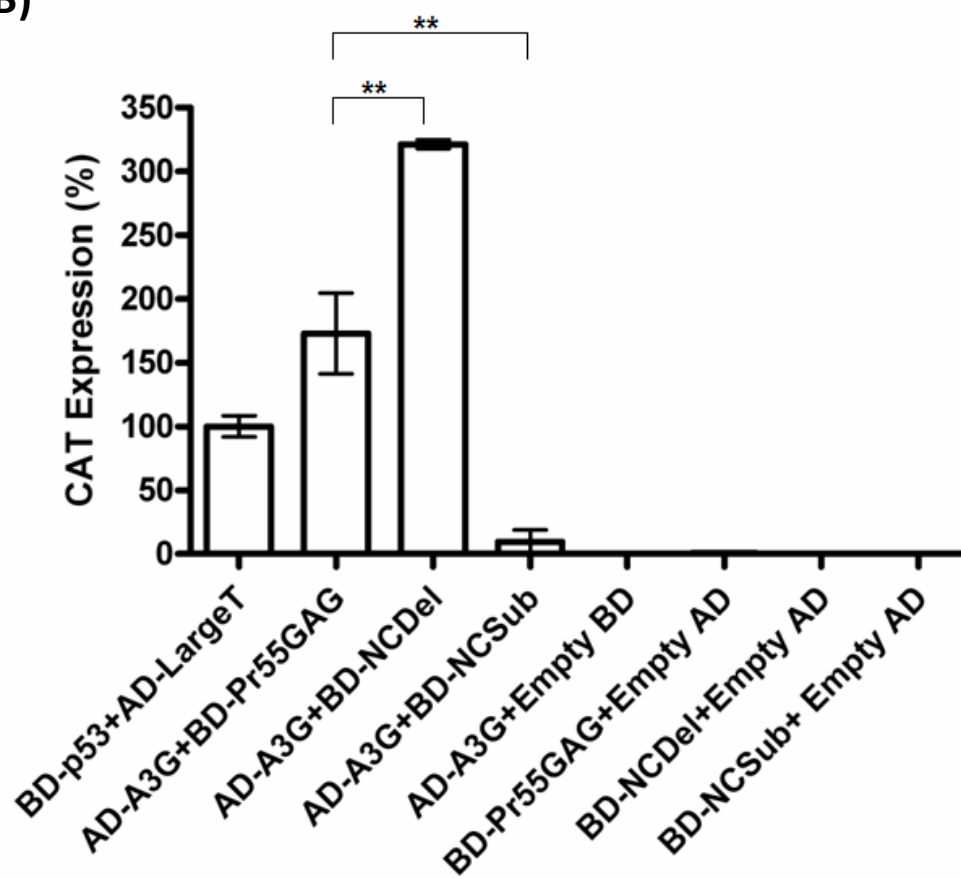
{A} Table showing the absolute CAT values obtained by CAT ELISA against the various combinations of plasmids used in a transfection. The positive control for the assay exploited the interaction between murine p53 and SV40 large T antigen. The negative controls for the assay were individual plasmids expressing AD/BD domain fused chimeric proteins of interest together with either empty BD or empty AD vector respectively.

{B} Bar chart depicting the CAT reporter expression levels relative to the positive and negative controls (the positive control being 100% and the lowest negative control being 0%) calculated using the absolute CAT values in Table 3.9 {A}. Error bars indicate Mean \pm SEM (n=2 experiments). **= significant difference (p<0.01). The p values were calculated using one way ANOVA with post test.

(A)

Sr No.	Plasmid combinations	Absolute CAT values (pg/ml)	
		Expt 1	Expt 2
1.	BD-p53 + AD-LargeT (Positive Control)	460	390
2.	AD-A3G +BD-Pr55 ^{GAG}	870	600
3.	AD-A3G + BD-NCDel	1380	1350
4.	AD-A3G + BD-NCSub	59	24
5.	AD-A3G + Empty BD	1	0
6.	BD-Pr55 ^{GAG} + Empty AD	7	0
7.	BD-NCDel + Empty AD	2	0
8.	BD-NCSub + Empty AD	2	0

(B)



3.7.2 Vif-Pr55^{GAG} interaction

When COS-1 cells were transfected with AD-Vif and BD-Pr55^{GAG} along with GFP reporter vector, positive GFP expression was observed similar to that with BD-p53 and AD-LargeT (positive control) (Fig. 3.10 A). Thus AD-Vif and BD-Pr55^{GAG} interact specifically with each other. Similarly when AD-Vif was transfected with BD-NCDel, positive GFP expression was observed indicating that the deleted region in NCDel did not play any part in the interaction of Vif with Pr55^{GAG} (Fig. 3.10 B). In contrast, when cells were transfected with AD-Vif and BD-NCSub, there was a huge decrease in reporter GFP expression signifying that the substitution mutations in NCSub were responsible for disrupting the interaction of Vif with Pr55^{GAG} (3.10 C).

Later, images were analysed using the software ImageJ for quantifying the change in GFP intensity observed. Figure 3.11 A tabulates the mean gray values of images against the various plasmid combinations employed. When a bar chart was plotted to demonstrate the relative GFP intensity by using the mean gray values in figure 3.11 A, the GFP intensity of AD-Vif and BD-Pr55^{GAG} was almost 2 times more to that observed with the positive control (Fig. 3.11 B). The GFP intensity of AD-Vif with BD-NCDel was even more than that observed with AD-Vif and BD-Pr55^{GAG} however this increase was not significant ($p>0.05$) (Fig. 3.11 B). In contrast there was a huge drop in GFP intensity observed with AD-Vif and BD-NCSub when compared to that of AD-Vif and BD-Pr55^{GAG}. This decrease in GFP intensity observed in comparison with AD-Vif and BD-Pr55^{GAG} was statistically significant ($p<0.001$). Thus mutations in the basic linker region significantly impair the Vif - Pr55^{GAG} interaction.

Figure 3.10 GFP reporter expression levels of the interactions between wild type Pr55^{GAG} and the two GAG mutants with Vif: COS-1 cells were transfected with the respective plasmids using a standard Lipofectamine transfection protocol detailed in section 2.5.3. Live cells were observed for GFP expression 48 hours post transfection under UV light microscope (section 2.7).

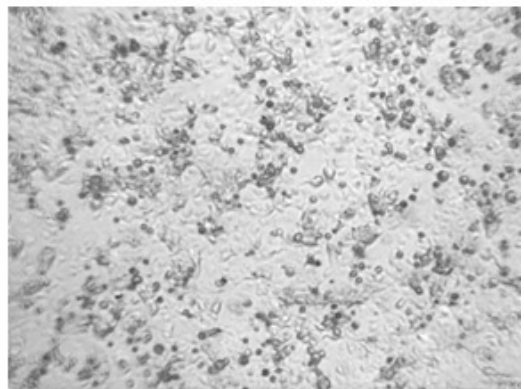
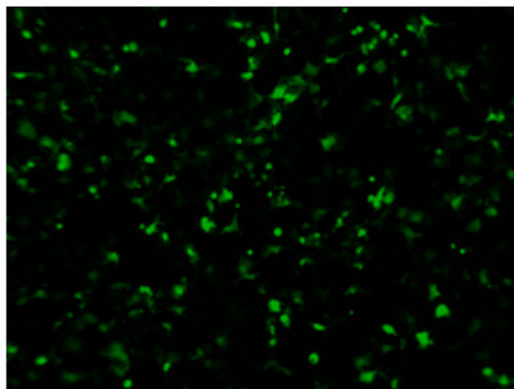
- {A} BD-Pr55^{GAG} + AD-Vif: Wild type Pr55^{GAG} and Vif together with GFP reporter
- {B} BD-NCDel + AD-Vif: NCDel mutant of Pr55^{GAG} and Vif together with GFP reporter
- {C} BD-NCSub + AD-Vif: NCSub mutant of Pr55^{GAG} and Vif together with GFP reporter

The levels of GFP reporter expression were monitored to determine whether an interaction was present between the two proteins of interest or not whereby positive GFP expression indicated the presence of an interaction while no or background level GFP expression indicated no interaction.

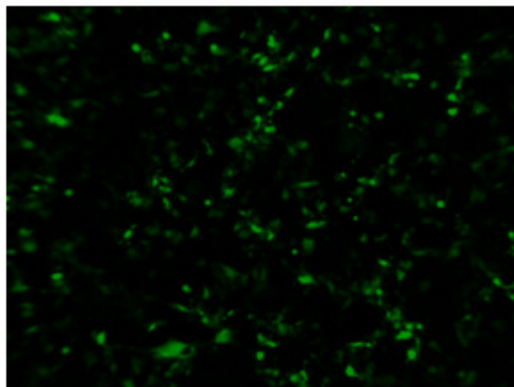
Panels on the right hand side show visible light image of the transfected cells and panels on the left display fluorescence of GFP under UV light.

(A)

40X Magnification



(B)



(C)

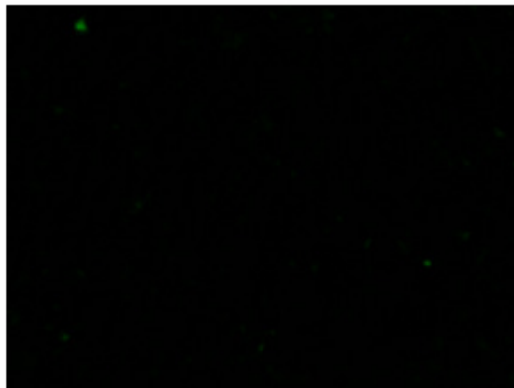


Figure 3.11 Quantifying the level of GFP expression using the image analysis software ImageJ to provide some indication of the strength of the interactions of Vif with wild type Pr55^{GAG} and the two GAG mutants: COS-1 cells were transfected with the indicated plasmid combinations in addition to the GFP reporter vector. 48 hours post-transfection, the cells were observed under UV light microscope and images were analysed for GFP expression using the image analysis software ImageJ. The RGB images were converted to gray scale to allow measurement of the gray values of each pixel within an image. The mean gray value of all pixels was thus directly proportional to the intensity of GFP.

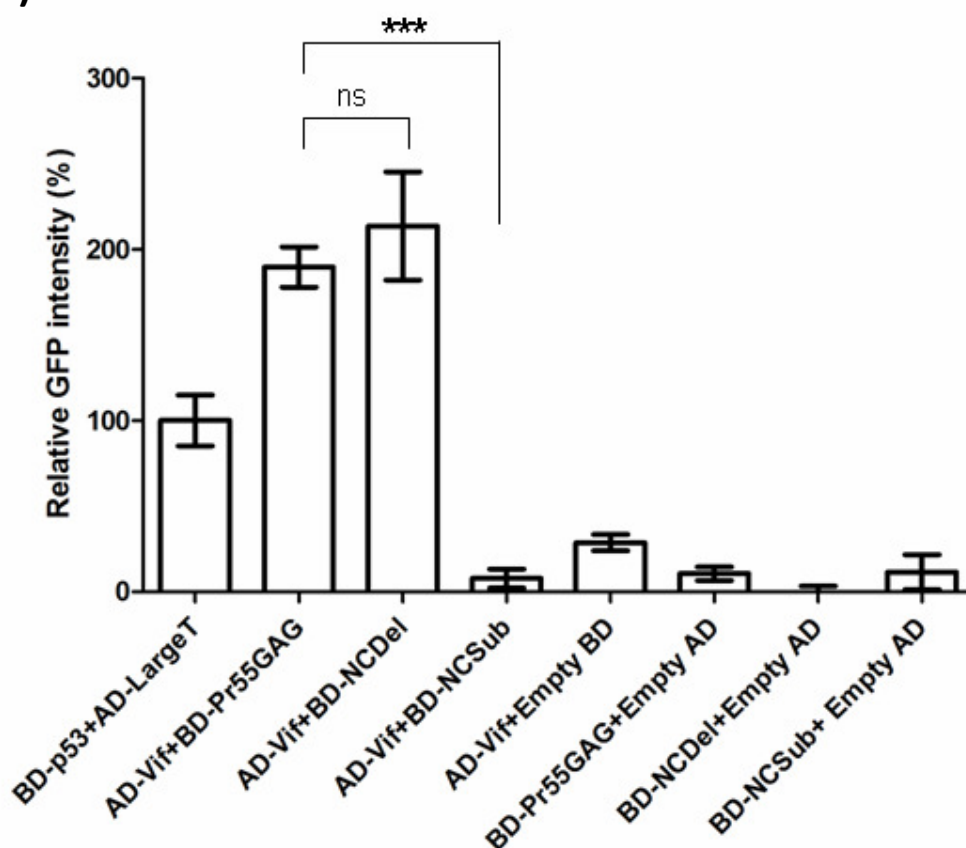
{A} Table depicting the mean gray values of images analysed using ImageJ against the plasmid combinations used in a transfection. The positive control for the assay exploited the interaction between murine p53 and SV40 large T antigen. The negative controls for the assay were individual plasmids expressing AD/BD domain fused chimeric proteins of interest together with either empty BD or empty AD vector respectively.

{B} Bar chart depicting the GFP intensity within an image relative to the positive and negative controls (the positive control being 100% and the lowest negative control being 0%) drawn using the mean gray values in the table 3.11 {A}. Error bars indicate Mean \pm SEM (n=2 experiments). ns= difference not significant ($p>0.05$); ***= significant difference ($p<0.001$). The p values were calculated using one way ANOVA with post test.

(A)

Sr. No.	Plasmid combinations	Mean gray value (GFP Intensity/pixel)	
		Expt 1	Expt 2
1.	BD-p53 + AD-LargeT (Positive Control)	2.123	1.627
2.	AD-Vif +BD-Pr55 ^{GAG}	3.561	3.170
3.	AD-Vif + BD-NCDeI	4.292	3.241
4.	AD-Vif + BD-NCSub	0.431	0.247
5.	AD-Vif + Empty BD (Negative control)	0.767	0.609
6.	BD-Pr55 ^{GAG} + Empty AD (Negative control)	0.452	0.320
7.	BD-NCDeI + Empty AD (Negative control)	0.263	0.158
8.	BD-NCSub + Empty AD (Negative control)	0.571	0.232

(B)



GFP reporter data was later corroborated by employing the CAT reporter vector instead of the GFP vector and repeating the same experiments again. Figure 3.12 A tabulates the absolute CAT values obtained by performing the CAT ELISA on transfected cell extracts against the various combinations of plasmids employed to study the Vif-Pr55^{GAG} interaction. Figure 3.12 B depicts a bar chart drawn from the values in figure 3.12 A showing the CAT enzyme expression of various plasmid combinations employed relative to the positive and negative controls. BD-p53 and AD-LargeT give positive CAT expression (positive control) (Fig. 3.12 B). When cells were transfected with AD-Vif and BD-Pr55^{GAG} along with CAT reporter vector the CAT enzyme expression observed was almost 2.5 times more compared to that with the positive control (Fig 3.12 B). Similarly the CAT expression observed with AD-Vif and BD-NCDel was even more compared to that seen with AD-Vif and BD-Pr55^{GAG}. However this increase was statistically not significant when compared with AD-Vif and BD-Pr55^{GAG} ($p>0.05$) (Fig. 3.12 B). In contrast there was a statistically significant drop in CAT expression observed with AD-Vif and BD-NCSUB when compared with AD-Vif and BD-Pr55^{GAG} ($p<0.001$) (Fig. 3.12 B). This significant decrease in CAT expression of AD-Vif with NCSUB signifies the importance of the basic linker region of the NC of Pr55^{GAG} and its critical role in the Vif-Pr55^{GAG} interaction.

Figure 3.12 CAT reporter expression levels of the interactions between wild type Pr55^{GAG} and the two GAG Mutants with Vif: COS-1 cells were transfected with the respective plasmids using the standard Lipofectamine transfection protocol described in section 2.5.3. Cell extracts were analysed for CAT enzyme expression 72 hours post-transfection using a CAT ELISA kit (Roche) according to the manufacturer's instructions (section 2.7).

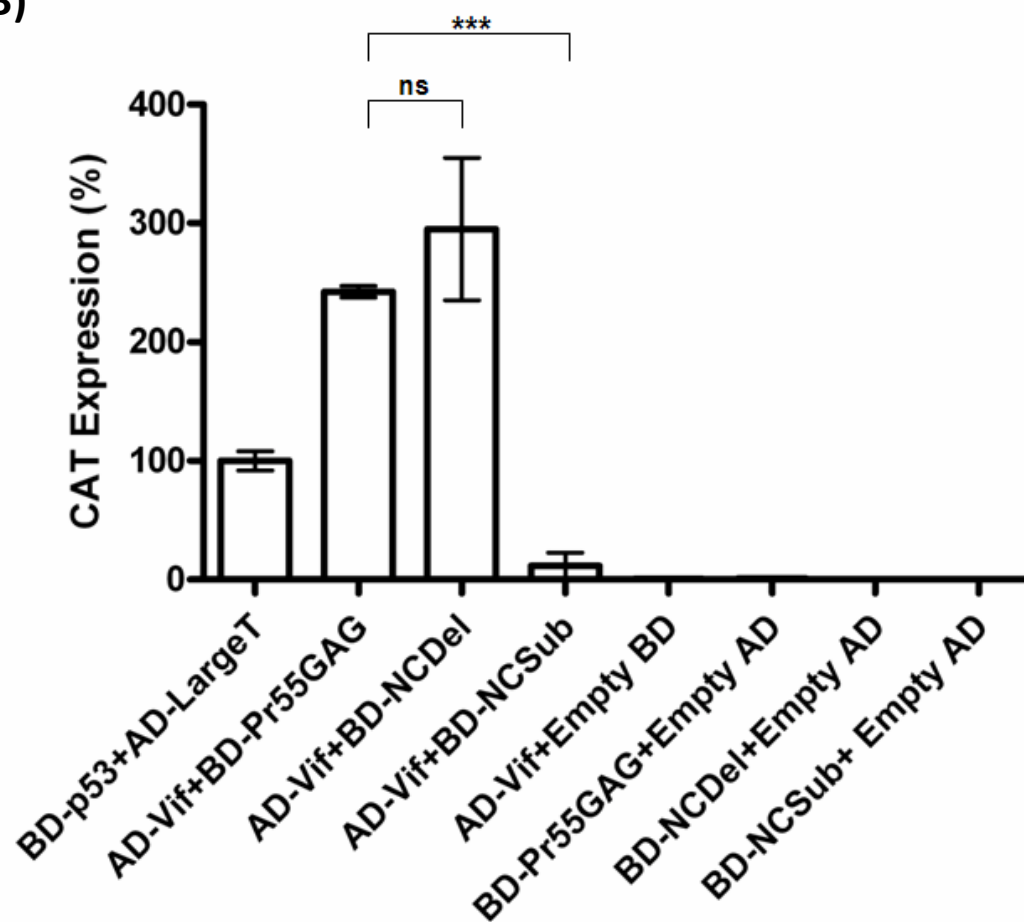
{A} Table showing the absolute CAT values obtained by CAT ELISA against the various combinations of plasmids used in a transfection. The positive control for the assay exploited the interaction between murine p53 and SV40 large T antigen. The negative controls for the assay were individual plasmids expressing AD/BD domain fused chimeric proteins of interest together with either empty BD or empty AD vector respectively.

{B} Bar chart depicting the CAT reporter expression levels relative to the positive and negative controls (the positive control being 100% and the lowest negative control being 0%) calculated using the absolute CAT values in Table 3.12 {A}. Error bars indicate Mean \pm SEM (n=2 experiments). ns= difference not significant (p>0.05); ***= significant difference (p<0.001). The p values were calculated using one way ANOVA with post test.

(A)

Sr No.	Plasmid combinations	Absolute CAT values (pg/ml)	
		Expt 1	Expt 2
1.	BD-p53 + AD-LargeT (Positive Control)	460	390
2.	AD-Vif +BD-Pr55 ^{GAG}	1050	1010
3.	AD-Vif + BD-NCDel	1510	1000
4.	AD-Vif + BD-NCSub	72	33
5.	AD-Vif + Empty BD	4	0
6.	BD-Pr55 ^{GAG} + Empty AD	7	0
7.	BD-NCDel + Empty AD	2	0
8.	BD-NCSub + Empty AD	2	0

(B)



Thus according to the GFP as well as CAT reporter data, there was a significant drop in reporter gene expression observed with both, AD-A3G and BD-NCSUB & AD-Vif and BD-NCSUB, suggesting that the mutated region within the basic linker of NC domain of Pr55^{GAG} is involved in the interaction of both Vif as well as A3G with Pr55^{GAG}.

3.8 Summary:

The mammalian two-hybrid assay studies previously performed in our lab have already confirmed the Pr55^{GAG}-A3G and Pr55^{GAG}-Vif interactions *in vivo* (Syed, 2008; Syed & McCrae, 2009). The BD-A3G plasmid was tested in the two-hybrid system in our lab before and was found to be self-activating reporter gene expression. Hence all the experiments were carried out using AD-A3G plasmid and hence could be carried out only in one way and not the other way round.

When the NCDel mutant of Pr55^{GAG} was tested for interaction with Vif/A3G using this assay, the results showed that deletion of amino acids 4-12 at the amino terminus of NC had no effect on the interaction with either Vif or A3G. On the contrary, an increase was observed in both GFP as well as CAT reporter expression when cells were transfected with either AD-A3G and BD-NCDel or AD-Vif and BD-NCDel when compared to AD-A3G and BD-Pr55^{GAG} or AD-Vif and BD-Pr55^{GAG} respectively. Important to note here is that the increase was not significant with AD-Vif and BD-NCDel in case of both GFP as well as CAT reporter data ($p > 0.05$) (Fig. 3.11 B and 3.12 B).

However in case of AD-A3G and BD-NCDel, there was a discrepancy observed between GFP and CAT reporter data. While in both cases there was an increase in reporter expression when compared with AD-A3G and BD-Pr55^{GAG}, the increase

observed with CAT reporter was significant ($p < 0.01$) (Fig. 3.9 B) while the increase seen with the GFP reporter data was not ($p > 0.05$) (Fig. 3.8 B). One possible reason for this discrepancy could be a difference in sensitivity of both these methods of analysis. While measurement for CAT enzyme requires an ELISA on the transfected cell extracts, the analysis of GFP expression is carried out by image analysis software on images of live cells expressing GFP.

If the CAT ELISA was a more sensitive method, then one possibility could be that the deletion of amino acids 4-12 from the N-terminal of the NC domain of Pr55^{GAG} changes the Pr55^{GAG} conformation in such a way that its affinity for A3G increases. On the other hand, if the GFP analysis data was correct and the increase was not significant, then the inference would be that the deletion has no effect on the A3G-Pr55^{GAG} interaction. However this is a very interesting finding and warrants further analysis.

By contrast, when the NCSub mutant of Pr55^{GAG} was used, significant reductions in reporter expression levels with both Vif and A3G were observed suggesting that the basic linker region between the two zinc finger motifs of the NC portion of Pr55^{GAG} is important for the interaction with both Vif and A3G. In case of A3G, this supports the conclusions reached by Burnett and Spearman (Burnett & Spearman, 2007).

Other possible interpretation of this result could be that the BD-NCSub mutant itself is non-functional and scrambled due to which it interacts neither with Vif nor with A3G. This possibility has been addressed in greater detail in the next chapter.

To summarise, both Vif and A3G interact at the same site on Pr55^{GAG} of HIV. This site is located in the basic linker region of its Nucleocapsid domain. These observations make possible a scenario where one of the actions of Vif in overcoming

the A3G block to virus replication is to compete with it for Pr55^{GAG} binding thereby preventing its encapsidation into the newly formed HIV virions (Fig. 3.8).

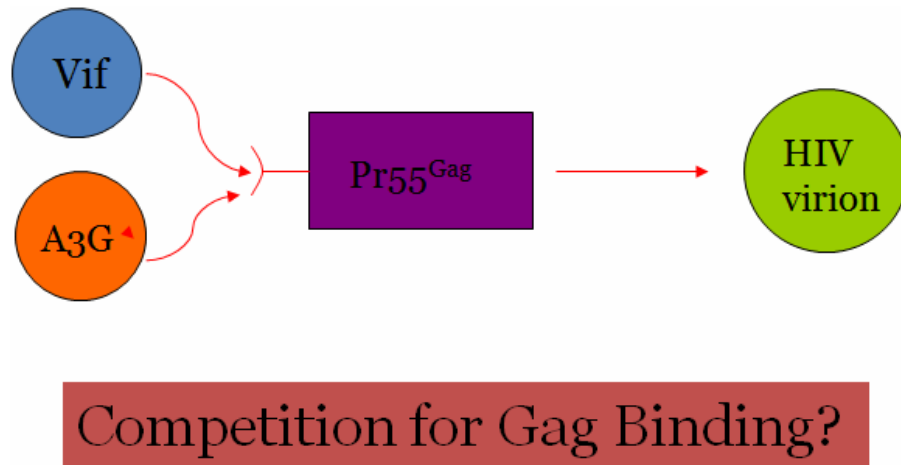


Figure 3.13 Diagrammatic summary of the results and the direction ahead

Vif and A3G both interact at the Basic Linker region of the Nucleocapsid domain of Pr55^{GAG} . As they interact at the same site on Pr55^{GAG} , a competition between them becomes possible. Thus Vif may successfully compete with A3G and prevent it from interacting with Pr55^{GAG} thereby excluding it from being incorporated into newly formed HIV virions.

CHAPTER 4

Assessment of the functionality of the

NCSub mutant of Pr55^{GAG}

In the previous chapter, Vif and A3G were both shown to interact at the same site on Pr55^{GAG}. However it remained possible that the negative interaction results for Vif and A3G binding to NCSub were due to misfolding of the mutated Pr55^{GAG}. To address this issue, a functionality test for the NCSub mutant of Pr55^{GAG} was required. This has been addressed in detail in this chapter.

4.1 Background

During the HIV-1 replication cycle, the interaction of the viral regulatory protein Rev with the Rev Response Element (RRE), its RNA target, is essential for the expression of the viral structural proteins (Cullen & Greene, 1989). Rev is known to facilitate nuclear export of RRE containing mRNAs and improve their stability.

It has however been found that in the absence of Rev, there is poor expression of viral proteins suggesting that there are some inhibitory sequences upstream of the structural protein coding sequence in the HIV genome (Schwartz et al, 1992b). Using site-specific mutagenesis, the inhibitory sequences have been successfully removed without requiring a change to the amino acid sequence of Pr55^{GAG}. This has resulted in the generation of a Pr55^{GAG} expression vector in which Pr55^{GAG} synthesis is independent of Rev (Schwartz et al, 1992a). This rev-independent Pr55^{GAG} expression construct directs formation of GAG virus-like particles (VLPs) and their release into the supernatant.

4.2 Plasmid used in the assay

The rev-independent GAG expression plasmid (pGAG-EGFP) directs the expression of GAG-EGFP fusion protein and allows the formation of GAG VLPs and their release into the supernatant (Fig. 4.1). This plasmid was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (Cat#11468) to which it was supplied by Dr. Marilyn Resh.

4.3 Incorporation of the same mutations in the pGAG-EGFP plasmid sequence as in NCSub mutant

Using a similar strategy to that described in section 3.5.2, MutGAG (GM) was constructed by site-directed mutagenesis of pGAG-EGFP (G) using PCR based mutagenesis with the forward primer 1551GagMutF and the reverse primer 1551GagMutR (Table 2.2.1). This produced a mutated Pr55^{GAG} in which two basic amino acids (Arginine and Lysine) had been substituted with two acidic ones (Glutamic acid) in the basic linker of the NC region of the protein similar to NCSub (Fig. 1.3 Appendix).

4.4 Hypotheses

4.4.1 Membrane localisation

One of the functional properties of Pr55^{GAG} expressed inside cells is to localise to the plasma membrane for initiating particle assembly (Bryant & Ratner, 1990). When

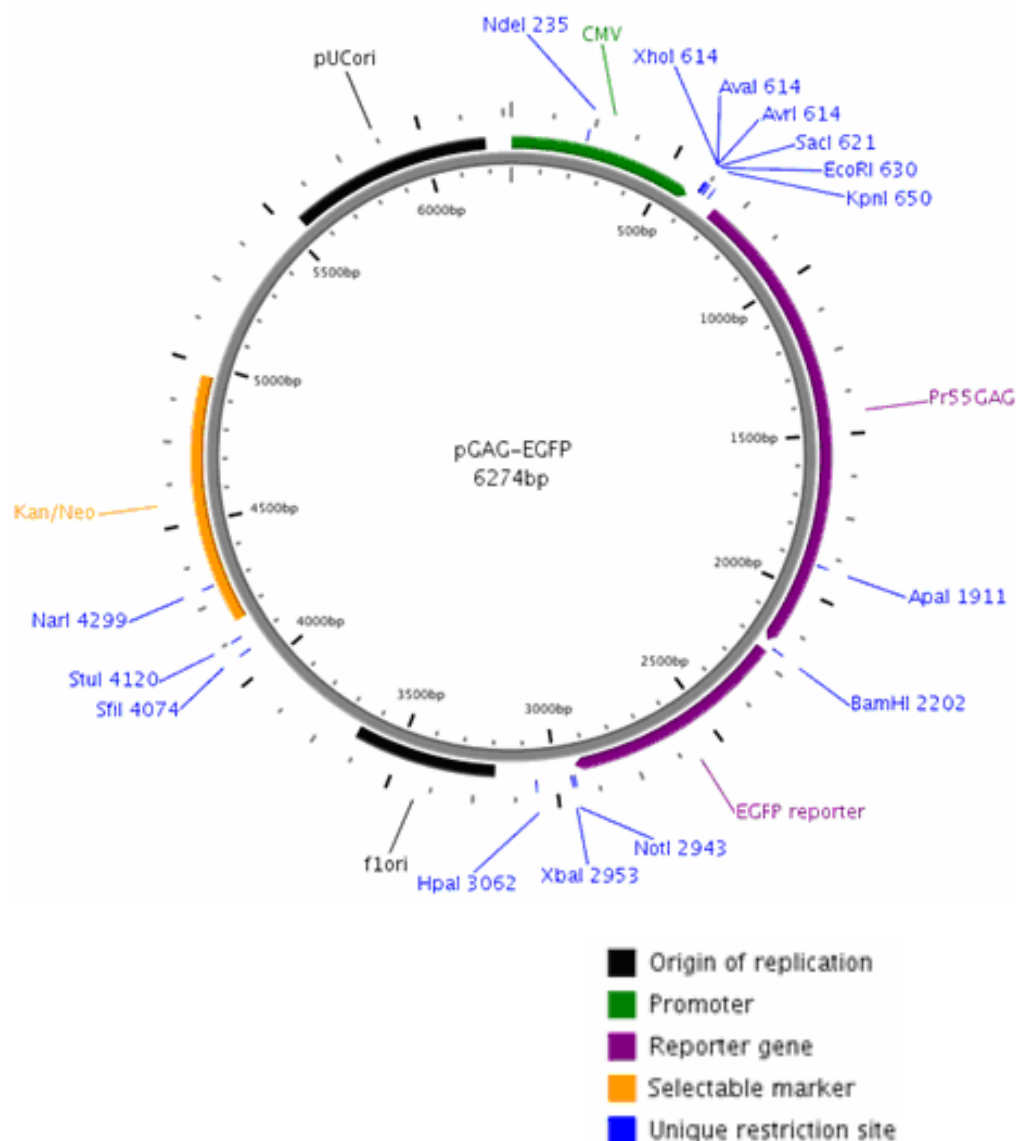


Figure 4.1 Vector used for the GAG assembly assay. pGAG-EGFP (G): This plasmid directs the expression of a Pr55^{GAG}-EGFP fusion protein from the CMV promoter and was originally constructed to allow imaging of the intracellular trafficking of the GAG protein. The expressed fusion protein also forms fluorescent GAG virus-like particles with equal efficiency to wild-type GAG.

the wild-type GAG plasmid (G) is transfected into 293T human embryonic kidney cells, the Pr55^{GAG}-GFP fusion protein localises to the cytoplasmic membrane. For the mutant Pr55^{GAG} (GM) to be functional, the expressed GM-GFP fusion protein should also localise to the plasma membrane similar to wild type GAG.

4.4.2 Particle assembly

The Gag expressed when wild-type GAG plasmid (G) is transfected into 293T cells, forms virus-like particles. If the mutant GAG protein (GM) folds correctly and is functional, it will also form virus-like particles similar to those produced by the wild type protein.

4.5 Assays employed:

4.5.1 Membrane localisation

To study the membrane localisation of the Pr55^{GAG}-GFP fusion protein, 293T cells were plated into 12 well dishes as detailed in section 2.9. The cells were separately transfected with 1.5µg of either G or GM plasmid using the standard Lipofectamine transfection protocol (section 2.5.3). The GFP-tagged wild type and mutant GAG fluorescent proteins were detected inside cells by using a confocal microscope as described in section 2.9.

4.5.2 Sucrose gradient analysis of GAG VLPs

The assay protocol for the sucrose gradient analysis of GAG VLPs has been described in detail in section 2.8 of Materials and Methods. Cells were transfected with 15µg of either G or GM plasmid using the calcium phosphate transfection

protocol described in section 2.5.4. (Campanero & Flemington, 1997). The VLPs were analysed as described in section 2.8.

4.6 Results

4.6.1 Confocal imaging of the expressed GAG-GFP fusion protein inside cells

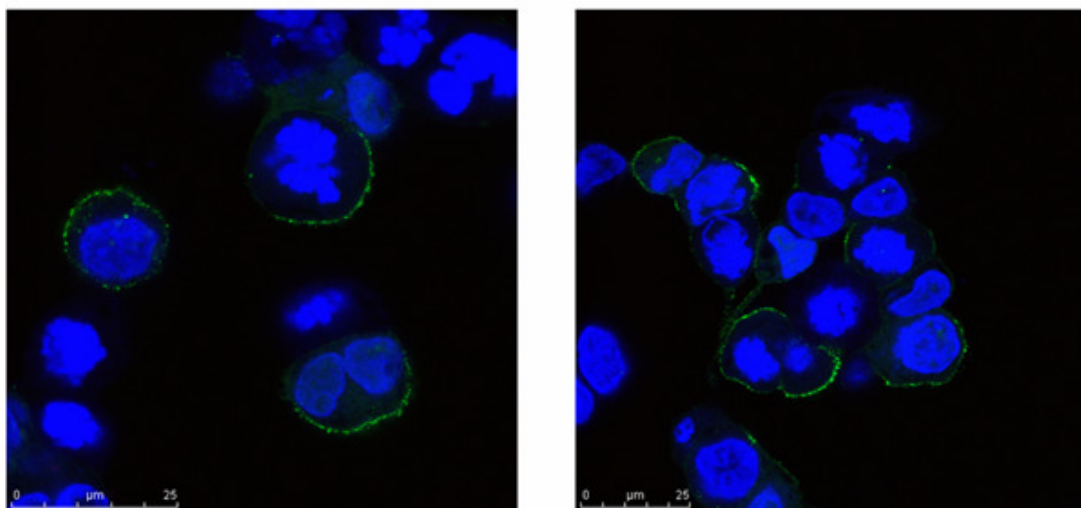
To study the localisation of GAG proteins inside cells, 293T cells were transfected with either G or GM plasmid and prepared for imaging as described in sections 4.5.1 and 2.9. The GFP fluorescence inside cells was observed under the confocal microscope.

Wild-type GAG-GFP fusion protein localised to the cytoplasmic membrane when 293T cells were transfected with the G plasmid (Fig.4.2). Similarly when cells were transfected with the GM plasmid, the mutant Pr55^{GAG}-GFP fusion protein also localised to the plasma membrane indicating that the mutant had the same functional property as wild-type Pr55^{GAG} (Fig. 4.2).

4.6.2 VLP assembly assay

To further test the functionality of the GM mutant of Pr55^{GAG}, 293T cells were separately transfected with either G plasmid expressing wild-type Pr55^{GAG} fused to GFP or the GM plasmid which expressed a mutated version of Pr55^{GAG} fused to GFP where the two basic amino acids (Arginine and Lysine) in the basic linker of the nucleocapsid were replaced with two acidic amino acids (Glutamic acids). 72 hours post-transfection the supernatant atop the transfected cells was collected and processed as detailed in section 2.8 (Fig. 4.3).

{A} Wild type Pr55^{GAG} (G)



{B} Mutant Pr55^{GAG} (GM)

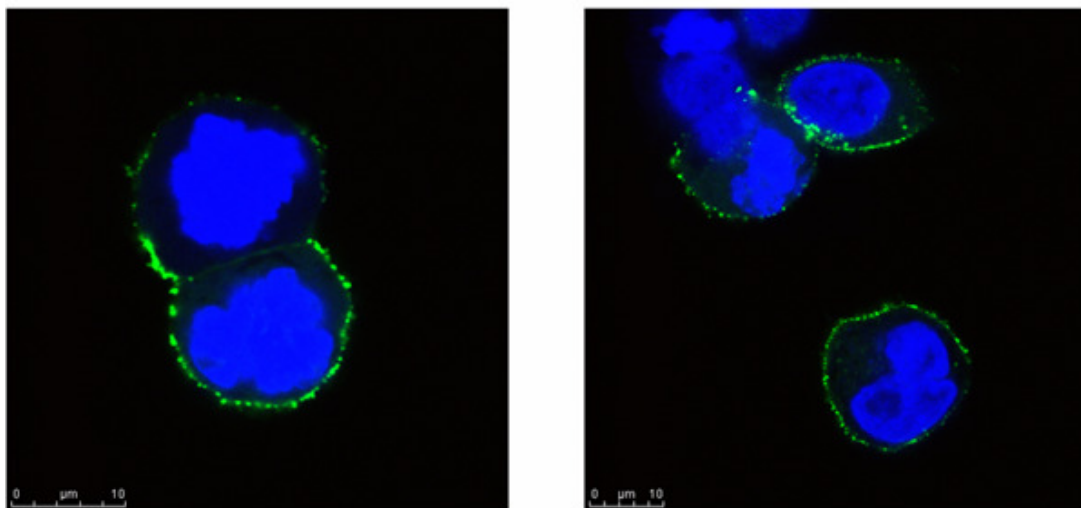


Figure 4.2 GFP localisation of wild type (G) and mutant (GM) GAG-GFP fusion proteins inside 293T cells studied using confocal microscope. 293T cells were separately transfected with either G or GM plasmid using the standard Lipofectamine transfection protocol described in section 2.5.3. 48 hours post-transfection, the cells were prepared for imaging under the confocal microscope as described in section 2.9. Green fluorescence indicates the position of the GAG-GFP fusion protein, with the nuclei of cells being stained blue due to the nuclear stain DAPI.

{A} Wild type Pr55^{GAG}-GFP fusion protein (scale bar is 25μm)

{B} Mutant Pr55^{GAG}-GFP fusion protein (scale bar is 10 μm)

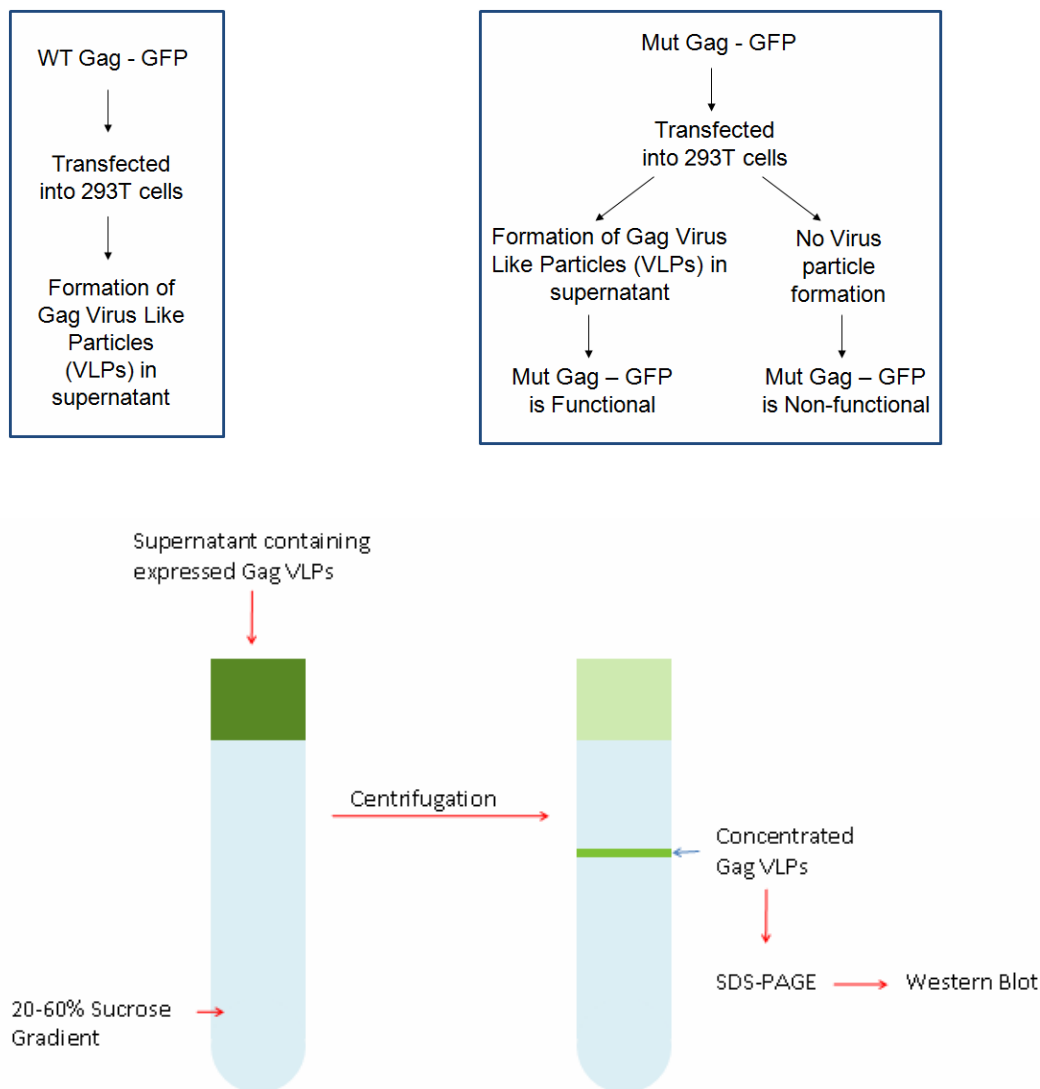


Figure 4.3 GAG assembly assay to test the functionality of the NCSub mutant of Pr55^{GAG}. The rationale of this assay is that if the mutant GM is able to form VLPs with similar efficiency and density to those produced by wild type G, then it is functional indicating that its folding has not been disrupted by the substitutions in the basic linker region of NC. 293T cells were transfected with either G/ GM plasmid using the calcium phosphate transfection protocol described in detail in section 2.5.4. Supernatant containing VLPs generated from the respective plasmids is concentrated and centrifuged through 20-60% sucrose gradients. Equal aliquots were collected from the bottom of the gradient, fractionated on SDS-PAGE gel and analysed by immunoblotting for the presence of GAG protein.

Alternate fractions of the gradient from aliquot number 2 to 14 together with the pellet fraction 'p' as described in section 2.8 were first analysed for the presence of GAG virus like particles. The expressed wild-type Pr55^{GAG} and the mutant Pr55^{GAG} both generated VLPs following transfection into 293T cells (Fig. 4.4). The VLPs were detected in aliquot number 4 of the gradient in both the wild type and the mutant plasmid transfections together with some diffusely stained smears in aliquots 6 to 10 with the smear being more prominent in aliquot number 10 (Fig. 4.4). Consequently it was decided to use only aliquots 4, 6, 8 and 10 for further studies on the GAG VLPs.

Figure 4.5 shows gradient fractions 4, 6, 8 and 10 re-analysed for the presence of GAG VLPs with both wild type and mutant GAG proteins. The VLPs were again found to be present in aliquot number 4 of the gradient fraction with wild type Pr55^{GAG}. The density of aliquot number 4 was later measured using a refractometer and found to be 1.16 g/ml. Pr55^{GAG} carrying the substitution mutations of NC seen in NCSUB was also able to generate VLPs in the GAG assembly assay, with VLPs being found in the same gradient fraction (aliquot number 4) as that seen for wild type GAG (Fig. 4.5). The density of fraction 4 containing mutant GAG VLPs was also measured and was found to be the same as that seen for wild type GAG VLPs (1.16 g/ml). There was also a smear of GAG protein detected on the blot corresponding to gradient fraction 8 (Fig. 4.5) which is discussed in detail in the next section.

The overall conclusion of this assay is therefore that the mutant GAG was able to generate VLPs which were found in the same gradient fraction as wild type GAG VLPs and had the same density as wild type Pr55^{GAG} VLPs. Thus the basic linker

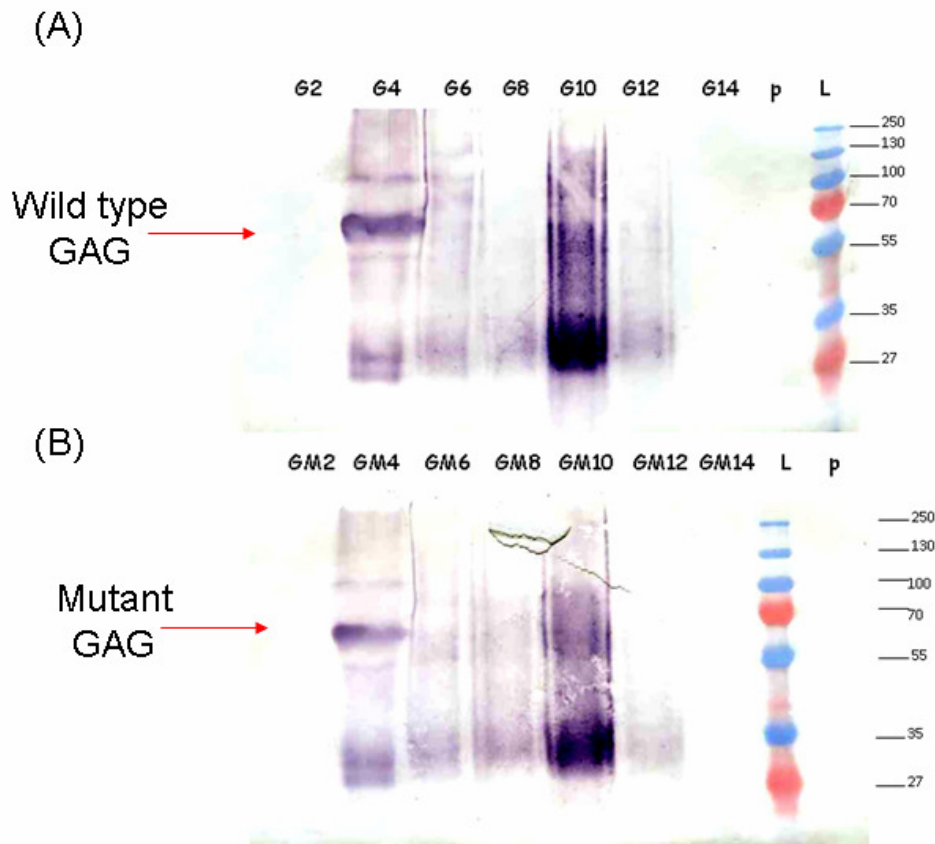


Figure 4.4 Sucrose gradient analysis of GAG VLPs. 293T cells were transfected with either G or GM plasmid separately using the standard calcium phosphate transfection protocol mentioned in section 2.5.4. Supernatant containing VLPs generated from the respective plasmids was concentrated and centrifuged through 20-60% sucrose gradients. Equal aliquots from 1 to 14 along with pellet fraction ‘p’ were collected from the bottom of the gradient. Alternate aliquots (p, 2, 4, 6 etc.) were fractionated on an SDS-PAGE gel and analysed by immunoblotting for the presence of GAG protein. Pr55^{GAG} was probed with a mouse anti-p24 antibody (1:3000) as described in section 2.2.4. Phosphatase conjugated goat anti-mouse was used as a secondary antibody(1:25,000). The blot was detected colorimetrically using BCIP/NBT alkaline phosphatase substrate tablets (Sigma-Aldrich B5655) dissolved in deionised water (section 2.6.3). The track labelling indicates the fraction number analysed with ‘L’ being the protein ladder. The numbering down the right side shows the migration position of the molecular weight size markers in KDaltons. The migration positions of wild type Gag (blot A) and the GAG mutant (blot B) in fraction number 4 (G4 and GM4 respectively) are indicated on the left hand side of the blot.

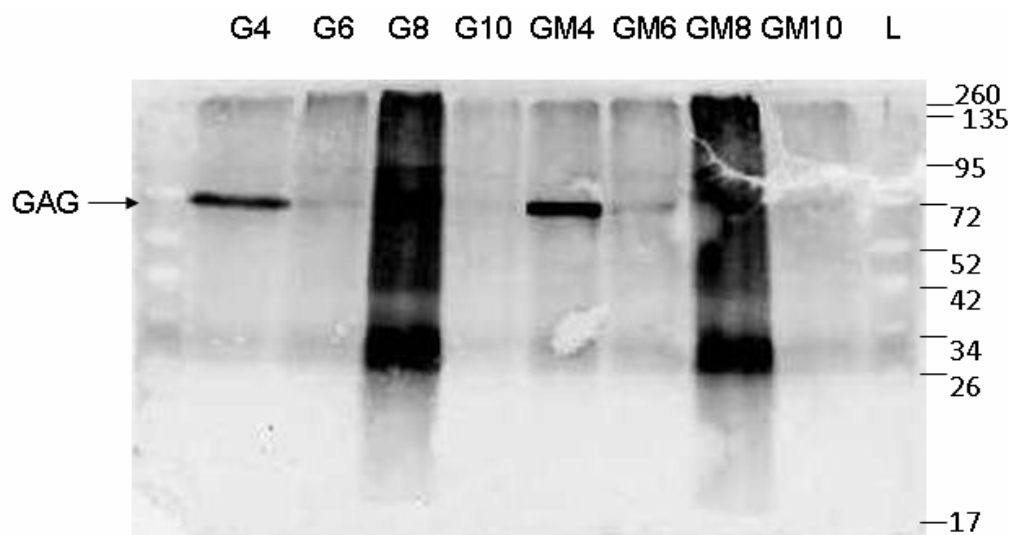


Figure 4.5 VLP assembly assay of the NCSub mutant of Pr55^{GAG}. Wild type(G) and mutant (GM) GAG VLPs were fractionated on 20-60% sucrose gradients and gradient fractions analysed by polyacrylamide gel electrophoresis and western blotting using anti-p24 as the primary antibody as described in section 2.2.4. Peroxidase conjugated goat anti-mouse (1:5000) was used as the secondary antibody. Proteins were detected using an ECL Chemiluminescence Detection Kit (GE Healthcare Life Sciences) and analysed using the Bio-Rad ChemiDoc XRS Imaging System. The numbering of the gel tracks denotes the specific sucrose gradient fraction analysed with L being the protein ladder and the numbering down the right side shows the migration position of molecular weight size markers in KDaltons. The position of wild type and mutant Pr55^{GAG} in gradient fraction G4 and GM4 respectively is arrowed.

Pr55^{GAG} mutant was assembling correctly indicating that the mutations in the basic linker of its nucleocapsid domain did not have any effect on the Pr55^{GAG} folding.

4.7 Summary

It has been well established that Pr55^{GAG} can direct the synthesis of virus-like particles (VLPs) both *in vivo* and *in vitro* without any requirement for other accessory viral or cellular factors (Campbell & Rein, 1999; Campbell & Vogt, 1995). However, these VLPs are non-infectious due to the absence of viral RNA.

Each VLP is composed of ~5000 GAG molecules forming a compact structure as a result of the ordered multimerisation of individual GAG proteins (Briggs et al, 2004). A small change at the amino acid level in Pr55^{GAG} could potentially result in improper protein folding and ultimately prevent the multimerization of GAG molecules.

The fact that mutant GAG was found localised to the cytoplasmic membrane similar to wild type GAG and formed VLPs with similar efficiency and density to those of the wild type protein is a clear demonstration that the amino acid substitutions made to produce the NCSub mutant had not lead to a large change in folding, allowing the protein to retain its functionality.

However the plasmid used in this assay was a rev-independent codon optimised version of Pr55^{GAG} fused to GFP (pGAG-EGFP) whereas the plasmid used in the mammalian two-hybrid studies detailed in the previous chapter was Pr55^{GAG} fused to the DNA binding domain (BD-Pr55^{GAG}). Although the two versions of Pr55^{GAG} used in the experiments described in chapters 3 and 4 were therefore different, it could still be argued that the mutations in the basic linker region did not compromise

the ability of the Pr55^{GAG} protein to self-assemble into virus like particles thus retaining its functionality. A more direct approach would have been to subclone the Pr55^{GAG}-DNA binding domain sequence into a CMV promoter vector and express the Pr55^{GAG} fusion protein in the presence of the viral Rev protein (Rev dependent Pr55^{GAG} expression). However this was technically more demanding and hence it was decided to insert the mutations into the codon optimised version of Pr55^{GAG} and analyse the VLPs obtained from this construct.

In addition to the GAG band detected in aliquot number 4 with both the wild type and mutant GAG proteins, there was also a smear of GAG protein detected in aliquot number 10 (Fig. 4.4) and aliquot number 8 (Fig. 4.5). The smear was darker especially in the ~25kDa region of the blot. This smear was most probably due to mechanical fragmentation of GAG VLPs occurring during the various steps involved in analysis. This fragmentation presumably affected the density of the VLPs resulting in the fragmented proteins being detected in the upper aliquots of the gradient corresponding to the less dense sucrose fractions. The reason for the smear being more prominent in two different aliquot fractions (fraction 10 and fraction 8 in Fig. 4.4 and 4.5 respectively) in two separate experiments could be due to the instrumental error introduced while preparing the sucrose gradient. However the changes observed were the same for both G as well as GM proteins obviating the error and hence allowing for a rational comparison of the results obtained.

Another attractive approach which could have been applied to tackle the functionality issue of GAG VLPs was to compare the *in vitro* proteolytic digestion profiles of wild type and mutant GAG VLPs. The purified mutant and wild type GAG VLPs could be treated with HIV-1 Protease and subjected to SDS-PAGE followed by immunoblotting with mAb to p24 (AG 3.0) which enables the detection

of polyprotein p55, processing intermediates as well as mature p24. If their digestion profiles were found to be similar, it could be reasonably argued that their assembly was also not very different from each other. However, at that time, the sucrose gradient analysis of virus-like particles seemed a relatively simpler approach to adopt because of the ready availability of all the reagents needed for the experiment.

To summarise, inserting the same basic linker region mutations as those seen in NCSUB into the Rev-independent Pr55^{GAG} expression construct did not have any effect on its self-assembly suggesting that the mutations did not affect GAG multimerisation. Hence it can be reasonably argued that insertion of these mutations does not affect Pr55^{GAG} functionality.

CHAPTER 5

***In vivo* competition studies between Vif and**

A3G for Pr55^{GAG} binding

5.1 Introduction

Having successfully demonstrated that both Vif and A3G interact at the same site on Pr55^{GAG} and also that mutations in the basic linker of NC did not impact Pr55^{GAG} functionality, the logical next step was to investigate whether Vif and A3G compete with each other for this interaction site on Pr55^{GAG}. To do this a novel modification of the mammalian two-hybrid assay was developed.

5.2 Modification of the mammalian two-hybrid assay to study competition

In the classical mammalian two-hybrid assay, the two proteins between which the interactions are to be studied are fused in-frame to the DNA-binding domain (BD) and Activation domain (AD) of the two-hybrid assay vectors respectively. A positive interaction between these proteins brings the BD and AD domains into close proximity to each other thereby reconstituting the transcription factor and initiating reporter gene transcription. This assay has been explained in detail in section 3.4.

The novel modification to this assay introduced in this study was to express a third protein in addition to the pair of chimeric proteins mentioned above. This third protein had the capability to interact with one of the proteins within the pair; the only significant difference being that its interaction with its partner did not stimulate reporter gene transcription. Two hypothetical scenarios are possible in this case. If the interaction of the third protein with one of the proteins within the pair is mutually exclusive of the interaction between the pair, then expression of the third protein would have no effect on reporter gene transcription levels in the mammalian two-hybrid assay. On the other hand, if the third protein competes for interaction and has

a higher relative affinity for this interaction compared to the other component of the pair, this should result in a drop in reporter gene transcription levels as the interaction between the chimeric protein-pair is disrupted due to the competition.

AD-A3G interacts with BD-Pr55^{GAG} to give positive reporter gene expression in the two-hybrid assay. If interaction between Vif and Pr55^{GAG} has a higher affinity than that between A3G and Pr55^{GAG} then expressing additional BD-Vif in the two-hybrid assay, that is able to interact with the BD-Pr55^{GAG} fusion but not trigger reporter expression, should displace AD-A3G from the two-hybrid interaction and thereby lower reporter gene expression (Fig.5.1). It is important to note here is that AD-A3G and BD-Vif have already been shown not to interact with each other in a two-hybrid assay and therefore do not stimulate any reporter gene expression (Syed, 2008).

Thus expressing BD-Vif in an assay being driven by the AD-A3G - BD-Pr55^{GAG} interaction should allow a comparison to be made of the relative affinity of both these proteins for Pr55^{GAG} binding.

5.3 Assay protocol

The mammalian two-hybrid assay transfection protocol has been explained in section 2.7 of Materials and Methods. Increasing amounts of the BD-Vif plasmid (0ng to 1500ng) were co-transfected, together with 1500ng each of AD-A3G, BD-Pr55^{GAG} and the CAT reporter plasmids. As a negative control, the same experiment was performed in the reverse direction i.e. increasing amounts (0ng to 1500ng) of AD-A3G plasmid were co-transfected, together with BD-Vif, AD-Pr55^{GAG} and the CAT reporter plasmids. The overall amount of plasmid DNA in each transfection was balanced using an empty Activation Domain (AD) vector to eliminate any effects of

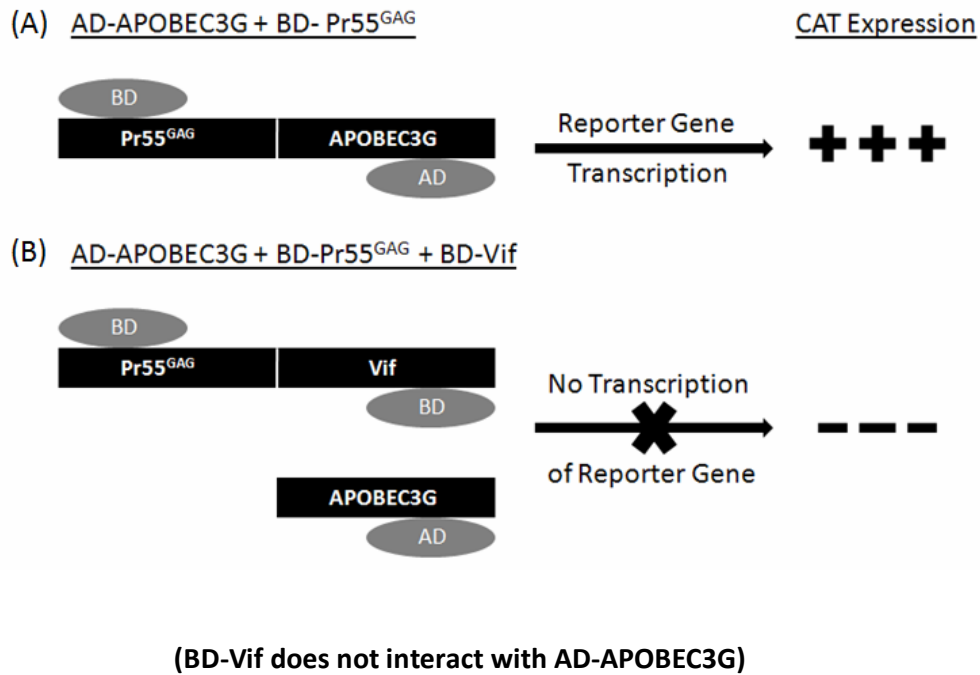


Figure 5.1 Simplified diagrammatic representation of the modified mammalian two-hybrid assay to study the competition between Vif and A3G for Pr55^{GAG} binding. (A) Interaction between AD-A3G and BD-Pr55^{GAG} gives positive CAT reporter expression levels (B) Addition of the plasmid BD-Vif to the system in (A) results in displacement of AD-A3G from BD-Pr55^{GAG} pushing the Activation Domain (AD) and Binding Domain (BD) away from each other resulting in loss of CAT expression. It has already been shown that BD-Vif does not interact with AD-APOBEC3G in this system and hence does not stimulate reporter gene expression (Syed, 2008).

varying the total amount of DNA used on reporter expression. Empty AD vector was used instead of the empty BD vector because BD vector is sometimes known to non-specifically activate reporter gene expression on its own which results in higher background reporter expression levels. CAT reporter expression was assayed as described in section 2.7. As BD-A3G was known to self-activate reporter gene expression (section 3.5.1), the experiments described in this study were designed only with AD-A3G and correspondingly the other plasmids used had to be modified. Hence for an assay driven by AD-A3G and BD-Pr55^{GAG}, increasing amounts of BD-Vif had to be added and when increasing amounts of AD-A3G were used in the control assay, the assay had to be driven by using the BD-Vif and AD-Pr55^{GAG} interaction.

5.4 Results

5.4.1 Effect of increasing BD-Vif levels in two-hybrid assays driven by an AD-A3G - BD-Pr55^{GAG} interaction

Figure 5.2 (A) tabulates the absolute CAT reporter expression values obtained against the various combinations of plasmids transfected. When COS-1 cells were transfected with AD-A3G and BD-Pr55^{GAG} together with CAT reporter vector in the absence of added Vif (BDVif-0ng), high CAT enzyme levels were observed (Fig. 5.2 A). This A3G-Pr55^{GAG} interaction acted as the positive control for the assay.

When BD-Vif and BD- Pr55^{GAG} were transfected into COS-1 cells together with empty AD vector, background levels of reporter gene expression were observed (Fig. 5.2 A). Similarly there was a background level of CAT expression detected when BD-Vif and AD-A3G were transfected together with empty AD vector indicating

that BD-Vif does not interact with AD-A3G (Fig. 5.2 A). It can therefore be inferred that these plasmid combinations or any individual plasmid within this combination did not activate reporter gene expression. These samples constituted the negative controls employed in this assay.

When the levels of BD-Vif were increased gradually from 0ng to 1500ng in an assay driven by the A3G-Pr55^{GAG} interaction, a corresponding stepwise reduction in CAT reporter levels was observed (Fig. 5.2 A).

The bar chart plotted to tabulate this change observed in the CAT reporter levels relative to the positive and negative controls showed that there was a significant reduction ($p < 0.01$) in CAT reporter expression when 750ng of BD-Vif was added to the assay driven by AD-A3G and BD-Pr55^{GAG} (Fig. 5.2 B). The CAT levels decreased even further in response to increased levels of BD-Vif with a very significant reduction ($p < 0.001$) observed compared to the positive control when 1500ng of BD-Vif was added to the assay driven by AD-A3G and BD-Pr55^{GAG} (Fig. 5.2 B).

5.4.2 Effect of increasing the levels of AD-A3G in two-hybrid assays driven by a BD-Vif - AD-Pr55^{GAG} interaction

To rule out the possibility that the results described in the previous section were due to non-specific effects, the same experiment was carried out in the reverse orientation. To do this, increasing levels of AD-A3G were added to a two-hybrid assay driven by BD-Vif and AD-Pr55^{GAG}. Figure 5.3 A tabulates the absolute CAT values obtained for the various combinations of plasmids employed in the experiment. BD-Vif and AD-Pr55^{GAG} resulted in positive CAT reporter expression in the absence of AD-A3G (AD-A3G- 0ng) (Fig. 5.3 A). As before, this represented the

Figure 5.2 CAT reporter expression levels for a two-hybrid assay driven by the AD-A3G & BD-Pr55^{GAG} interaction when the levels of BD-Vif plasmid co-transfected were increased from 0ng to 1500ng: COS-1 cells were transfected with plasmids using the standard Lipofectamine transfection protocol described in section 2.5.3. Cell extracts were analysed for CAT enzyme expression 72 hours post-transfection using a CAT ELISA kit (Roche) according to the manufacturer's instructions (section 2.7).

{A} Table showing the absolute CAT values obtained by CAT ELISA for the various combinations of plasmids used in a transfection. The concentration of BD-Vif was sequentially increased from 0ng to 1500ng balancing the total amount of DNA in each transfection with empty AD vector.

Z= AD-A3G +BD-GAG +CAT Reporter + pcDNA3.1-A3G-HA (1500ng each)

The positive control for the assay was

- Z + BD-Vif0 + EmptyAD1500 (Transfection in which the CAT reporter expression was driven by AD-A3G and BD-GAG in the absence of BD-Vif)

The negative controls for the assay were

- BD-Vif, BD-GAG and Empty AD vector (1500ng each) &
- BD-Vif, AD-A3G and Empty AD vector (1500ng each).

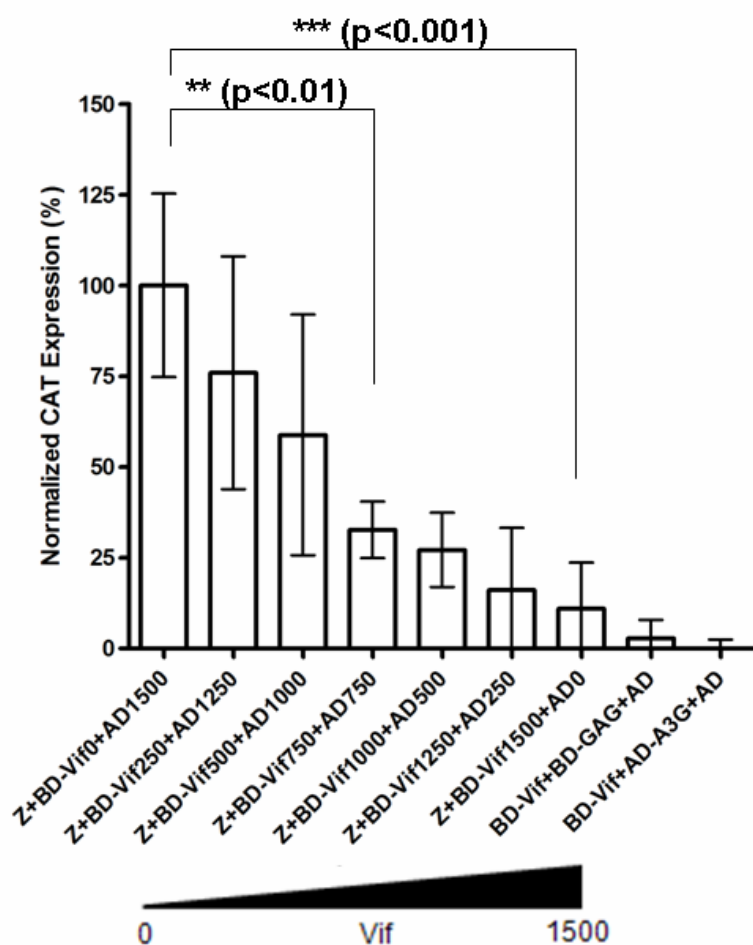
{B} Bar chart depicting the CAT reporter expression levels relative to the positive and negative controls (the positive control being 100% and the lowest negative control being 0%) calculated using the absolute CAT values in Table 5.2 {A}. Error bars indicate Mean \pm SEM (n=2 experiments). **= significant difference (p<0.01). ***= very significant difference (p<0.001). The p values were calculated using one way ANOVA with post test.

(A)

Sr No.	Plasmid combinations	Absolute CAT values (pg/ml)	
		Expt 1	Expt 2
1.	Z + BD-Vif0 + EmptyAD1500 (+ve ctrl)	1946	1277
2.	Z + BD-Vif250 + EmptyAD1250	1717	867
3.	Z + BD-Vif500 + EmptyAD1000	1505	627
4.	Z + BD-Vif750 + EmptyAD750	823	617
5.	Z + BD-Vif1000 + EmptyAD500	782	511
6.	Z + BD-Vif1250 + EmptyAD250	727	274
7.	Z + BD-Vif1500 + EmptyAD0	600	264
8.	BD-Vif + BD-GAG + Empty AD (-ve ctrl 1)	392	257
9.	BD-Vif + AD-A3G + Empty AD (-ve ctrl 2)	320	255

Z= AD-A3G+BD-GAG+CAT+pcDNA-A3G-HA

(B)



positive control for the assay. The negative controls used (1) AD-A3G + AD-GAG + empty AD and (2) AD-A3G + BD-Vif + empty AD both resulted in a background level CAT expression (Fig. 5.3 A).

In contrast to what was seen in the earlier competition experiment, in this case no stepwise reduction was observed when the levels of AD-A3G were increased from 0ng to 1500ng in an assay driven by the Vif-Pr55^{GAG} interaction (Fig. 5.3 A). When a bar chart showing the CAT values relative to the positive and negative controls was plotted, no significant change was observed ($p>0.05$) in the CAT reporter expression levels when the amount of A3G was increased gradually from 0 to 1500 ng (Fig. 5.3 B).

This result strongly supports the proposal that the step-wise decrease in CAT reporter expression observed with an increase in the levels of BD-Vif in an assay driven by the A3G-Pr55^{GAG} interaction (Fig. 5.2) was indeed specific.

5.5 Potential limitations of the competition assay

In addition to the competition hypothesis for Vif action, two other possible explanations for the results obtained in Figure 5.2 should be discussed. These are as follows:

5.5.1 Competition between BD-Vif and BD-Pr55^{GAG} for binding to the Upstream Activation Sequence of CAT reporter vector

The proposal here is that stepwise decrease in reporter gene expression observed with increasing BD-Vif levels in a two-hybrid assay driven by AD-A3G and BD-Pr55^{GAG} results from BD-Vif directly competing with BD-Pr55^{GAG} for binding to the specific Upstream Activation Sequence (UAS) in the promoter of the reporter vector

Figure 5.3 CAT reporter expression levels for a two-hybrid assay driven by the BD-Vif & AD-Pr55^{GAG} interaction when the levels of AD-A3G plasmid co-transfected were increased from 0ng to 1500ng: COS-1 cells were transfected with the respective plasmids using the standard Lipofectamine transfection protocol described in section 2.5.3. Cell extracts were analysed for CAT enzyme expression 72 hours post-transfection using a CAT ELISA kit (Roche) according to the manufacturer's instructions (section 2.7).

{A} Table showing the absolute CAT values obtained by CAT ELISA against the various combinations of plasmids used in a transfection. The concentration of AD-A3G was sequentially increased from 0ng to 1500ng balancing the total amount of DNA in each transfection with empty AD vector.

Z= AD-GAG +BD-Vif +CAT Reporter (1500ng each)

The positive control for the assay was

- Z + AD-A3G0 + EmptyAD1500 (Transfection in which the CAT reporter expression was driven by AD-GAG and BD-Vif in the absence of AD-A3G)

The negative controls for the assay were

- AD-A3G, AD-GAG and Empty AD vector (1500ng each) &
- BD-Vif, AD-A3G and Empty AD vector (1500ng each).

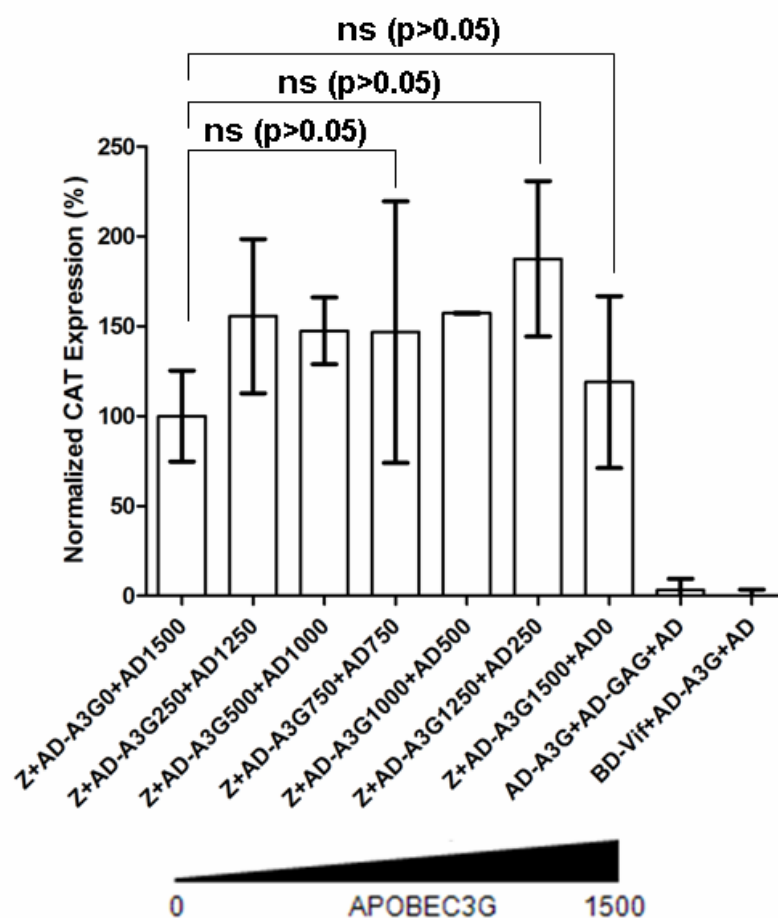
{B} Bar chart depicting the CAT reporter expression levels relative to the positive and negative controls (positive control being 100% and the lowest negative control being 0%) calculated using the absolute CAT values in Table 5.3 {A}. Error bars indicate Mean \pm SEM (n=2 experiments). ns= difference not significant (p>0.05). The p values were calculated using one way ANOVA with post test.

(A)

Sr No.	Plasmid combinations	Absolute CAT values (pg/ml)	
		Expt 1	Expt 2
1.	Z + AD-A3G0 + EmptyAD1500 (+ve ctrl)	1308	876
2.	Z + AD-A3G250 + EmptyAD1250	1932	1200
3.	Z + AD-A3G500 + EmptyAD1000	1656	1338
4.	Z + AD-A3G750 + EmptyAD750	2112	870
5.	Z + AD-A3G1000 + EmptyAD500	1578	1584
6.	Z + AD-A3G1250 + EmptyAD250	2208	1470
7.	Z + AD-A3G1500 + EmptyAD0	1662	846
8.	AD-A3G + AD-GAG + Empty AD (-ve ctrl 1)	320	212
9.	BD-Vif + AD-A3G + Empty AD (-ve ctrl 2)	268	209

Z= AD-GAG+BD-Vif+CAT

(B)



thereby displacing BD-Pr55^{GAG} binding thus squelching reporter gene expression by preventing its transcription initiation.

The assays described in sections 5.4.1 and 5.4.2 were all conducted by employing the empty AD vector to balance out the total DNA in all wells and eliminate any effects of varying total amount of DNA on transfection efficiency. The empty BD vector was not used because it is known to have a relatively higher rate of self-activation and gives a higher background signal as compared to empty AD vector. Nevertheless to address this possible explanation for the competition observed, the experiment described in section 5.4.1 was repeated balancing the total amount of DNA in each case with empty BD vector instead of empty AD vector.

The hypothesis would be that if the BD component of BD-Vif was indeed competing with BD-Pr55^{GAG} for the DNA-binding domain interaction site on the reporter vector, then the empty BD vector would also compete for the same site. The expectation would be that there should be a uniform decrease in CAT reporter expression across all samples as the total amount of DNA-binding domain vectors (empty BD + BD-Vif) in each sample would be the same (1500ng). However when this experiment was carried out a uniform decrease in CAT reporter expression was not observed but rather there was again a stepwise drop in CAT expression with increase in BD-Vif levels from 0ng to 1500ng (Fig. 5.4 A and B). This result shows that competition at the DNA-binding domain interaction site on the reporter vector was not the explanation for the observed competition of BD-Vif on the A3G-Pr55^{GAG} interaction.

Figure 5.4 CAT reporter expression levels for a two-hybrid assay driven by the AD-A3G & BD-Pr55^{GAG} interaction when the levels of BD-Vif plasmid co-transfected were increased from 0ng to 1500ng while the amount of total DNA in each transfection was balanced with empty BD vector: COS-1 cells were transfected with plasmids using the standard Lipofectamine transfection protocol described in section 2.5.3. Cell extracts were analysed for CAT enzyme expression 72 hours post-transfection using a CAT ELISA kit (Roche) according to the manufacturer's instructions (section 2.7).

{A} Table showing the absolute CAT values obtained by CAT ELISA against the various combinations of plasmids used in a transfection. The concentration of BD-Vif was sequentially increased from 0ng to 1500ng balancing the total amount of DNA in each transfection with empty BD vector.

X= AD-A3G +BD-GAG +CAT Reporter (1500ng each)

The positive control for the assay was

- X + BD-Vif0 + EmptyBD1500 (Transfection in which the CAT reporter expression was driven by AD-A3G and BD-GAG in the absence of BD-Vif)

The negative controls for the assay were

- BD-Vif, BD-GAG and Empty BD vector (1500ng each) &
- BD-Vif, AD-A3G and Empty BD vector (1500ng each).

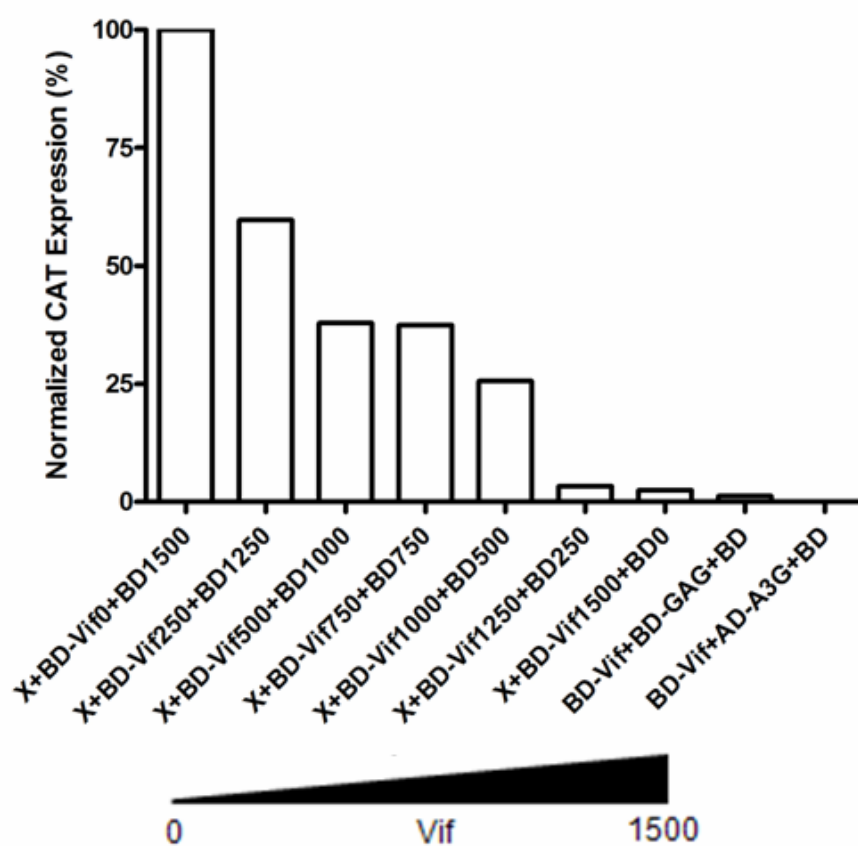
{B} Bar chart depicting the CAT reporter expression levels relative to the positive and negative controls (positive control being 100% and the lowest negative control being 0%) calculated using the absolute CAT values in Table 5.4 {A}.

(A)

Sr No.	Plasmid combinations	Absolute CAT values (pg/ml)
1.	X + BD-Vif0 + EmptyBD1500 (+ve ctrl)	1581
2.	X + BD-Vif250 + EmptyBD1250	1058
3.	X + BD-Vif500 + EmptyBD1000	774
4.	X + BD-Vif750 + EmptyBD750	767
5.	X + BD-Vif1000 + EmptyBD500	613
6.	X + BD-Vif1250 + EmptyBD250	324
7.	X + BD-Vif1500 + EmptyBD0	312
8.	BD-Vif + BD-GAG + Empty BD (-ve ctrl 1)	296
9.	BD-Vif + AD-A3G + Empty BD (-ve ctrl 2)	280

X= AD-A3G+BD-GAG+CAT

(B)



5.5.2 Proteasomal degradation of AD-A3G by BD-Vif

It has been clearly demonstrated that Vif expressed in the cytoplasm of HIV infected cells forms a Vif-Cul5-SCF complex which recruits A3G leading to its polyubiquitination and forwarding to the 26S Proteasome for degradation (Yu et al, 2003). Although the proteins expressed in the two-hybrid assays are targeted to the nucleus it remained possible that the reduced levels of CAT reporter seen in Fig. 5.2 resulted from increasing levels of BD-Vif promoting degradation of the AD-A3G synthesised in the assay.

To exclude this possibility, an additional plasmid, pcDNA3.1-A3G-HA, was also transfected into the COS-1 cells used in the competition assay (Figure 5.2). This plasmid expresses an HA-tagged A3G protein (A3G-HA) under the control of the constitutive immediate early promoter of cytomegalovirus. If the expressed BD-Vif was indeed promoting the degradation of AD-A3G resulting in decreased CAT enzyme expression, then it would be expected that it would also promote degradation of the HA-tagged A3G.

However Fig. 5.5 shows that when the level of BD-Vif expressing plasmid was increased from 0 to 1500ng, there was no change in the level of A3G-HA observed. As BD-Vif did not have any effect on the HA-tagged A3G, it can be reasonably argued that it would not have any effect on AD-A3G. These results therefore make it very unlikely that this explains the competition for binding seen in figure 5.2, serving to strengthen the main conclusion reached.

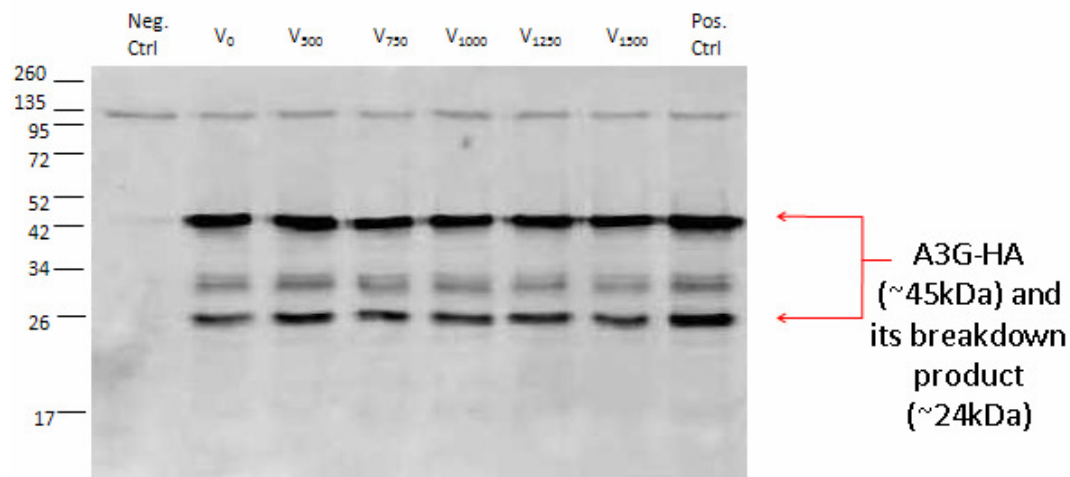


Figure 5.5 BD-Vif does not promote the degradation of A3G. Cell extracts were collected from the two-hybrid assays shown in Fig. 5.2 which were additionally transfected with a plasmid (pcDNA3.1-A3G-HA) that expresses an HA tagged A3G. Samples were electrophoresed on a 12.5% SDS-PAGE gel and transferred onto a nitrocellulose membrane as described in section 2.6. The western blot was probed with anti-HA antibody and signal detected by chemiluminescence as described in section 2.6.4. The track labelling indicates samples where increasing levels of BD-Vif were co-transfected V₀=0ng to V₁₅₀₀=1500ng. The positive control was produced from cells transfected solely with pcDNA3.1-A3G-HA and the negative control from cells where this plasmid was not added to the transfection. The migration positions of A3G-HA and a degradation fragment are indicated on the right hand side of the blot.

5.6 Conclusions

Increasing levels of Vif in the A3G-Pr55^{GAG} driven assay was found to squelch reporter gene expression in a concentration dependent fashion (Fig. 5.2 and section 5.4.1). By contrast, in an experimental control, increasing amounts of A3G in a Vif-Pr55^{GAG} driven assay had no effect on reporter expression levels (Fig. 5.3 and section 5.4.2). This demonstrated that the stepwise reduction of CAT expression with increasing levels of Vif was a specific phenomenon.

There are a number of alternative interpretations which could be made of these data. The first is that as the levels of BD-Vif are increased in the system, it competes with BD-Pr55^{GAG} for the UAS on the CAT reporter vector and hence squelches reporter gene expression due to the displacement of BD-Pr55^{GAG} away from the reporter vector (section 5.5.1). To counter this interpretation, empty BD vector was used to balance the total amount of DNA in each sample and the experiment shown in figure 5.2 repeated. This showed that the empty BD vector had no effect on the level of CAT reporter expression demonstrating that competition at the promoter of the reporter vector was not the explanation for the competition observed (Fig. 5.4 and section 5.5.1).

The second argument that can be raised against the proposed competition between Vif and A3G for Pr55^{GAG} binding was that as the levels of BD-Vif were increased in the system, the expressed BD-Vif was promoting the degradation of AD-A3G and this decrease in AD-A3G levels was leading to a drop in CAT reporter expression (section 5.5.2). To circumvent this argument, an additional plasmid expressing HA-tagged A3G was added to the transfections and the effect of increasing amounts of Vif on the levels of A3G-HA were detected. There was no change in the level of

A3G-HA in any of the samples indicating that BD-Vif did not promote the degradation of A3G-HA (Fig. 5.5). It is however the case that A3G-HA is expressed in the cytoplasm of transfected cells while the two-hybrid assay proteins localise to the nucleus. Nevertheless degradation of A3G by Vif is known to occur in the cytoplasm and nuclear degradation of A3G has not yet been reported. Hence it can be reasonably argued that BD-Vif does not degrade AD-A3G. Direct monitoring of the level of AD-A3G within a nuclear fraction would have been a more accurate way to get around this argument. However previous experience in the lab has shown that the expression levels of two-hybrid assay proteins are so low that they are very difficult to detect on western blots. It is this experience that prompted the use of a tagged version of A3G under the control of the CMV promoter. A more accurate experimental control would have been to incorporate a nuclear localisation signal into the A3G-HA used which would have enabled a correct estimation of the A3G dynamics within the nucleus.

To conclude, the results obtained in this competition assay strongly support the proposal that Vif can outcompete A3G for Pr55^{GAG} binding. The advantage of this two-hybrid based assay is that it is an *in vivo* assay and tries to mimic as closely as possible the environment within cells. Its inherent weakness lies in the expression levels of the two-hybrid assay proteins making difficult the direct monitoring forcing reliance solely on the read-out obtained from the reporter gene vector.

Clearly additional experiments using an independent assay system are necessary to support the conclusions reached here. However this finding opens up a new avenue to account for the mechanism by which Vif is able to overcome the block to HIV replication exerted by the A3G component of the innate immune response.

Chapter 6

Application of the GAG VLP assembly assay for competition studies between Vif and A3G

6.1 Introduction

Further to the findings that Vif and A3G compete with each other for interaction with Pr55^{GAG}, the next logical aim was to validate this competition using an independent experimental assay protocol. The sucrose gradient assay has already been discussed in Chapter 4 where it was employed to study the functionality of the NCSUB mutant of Pr55^{GAG}. In this section, the assay protocol was slightly modified to give readout of the competition between Vif and A3G for Pr55^{GAG} binding.

6.2 Background

The degradation resistant A3G variant has been described in previous reports (Opi et al, 2007). This mutant of A3G which cannot be degraded by HIV-1 Vif was employed to explore whether competition is an additional mechanism for Vif action in addition to the widely accepted mechanism of proteasomal degradation of A3G by Vif.

When expressed in cells, Pr55^{GAG} protein directs the formation of GAG VLPs in the supernatant. Both Vif and A3G are known to become incorporated into the newly formed virus particles through a specific interaction with Pr55^{GAG} (Bouyac et al, 1997a; Cen et al, 2004; Huvent et al, 1998; Schafer et al, 2004; Syed & McCrae, 2009).

In a scenario where both Vif and A3G are co-expressed in cells together with Pr55^{GAG}, the expressed Vif would deplete the cellular levels of A3G by proteasome-mediated degradation of the latter (Yu et al, 2003). Consequently there would be

very low amounts of A3G protein within the cells. If proteasomal degradation is considered as the primary mechanism of Vif action, then the small amount of A3G remaining would not be detectably incorporated into the GAG VLPs generated by the expressed Pr55^{GAG}. Thus the resultant VLPs would only have Vif detectably incorporated into them. With Vif-mediated degradation of A3G masking all other functions, it becomes very difficult to study if any additional mechanisms are employed by Vif to bypass the A3G block to virus replication.

To circumvent this difficulty, the degradation resistant A3G variant was used. The hypothesis underpinning its use was that if competition and not degradation was an important mechanism for Vif action, then despite of not being able to degrade this A3G mutant, Vif would still be able to compete with it and prevent its incorporation into the budding GAG VLPs (Fig. 6.1).

6.3 Plasmids used in the assay

6.3.1 pGAG-EGFP (G)

The Rev independent Pr55^{GAG} expression construct pGAG-EGFP (G) has already been described in chapter 4. This plasmid directs the formation of GAG VLPs in the supernatant when transfected in 293T cells.

6.3.2 pcDNA3.1-A3G-HA (A)

The 3xHA-tagged A3G expression plasmid pcDNA3.1-A3G-HA (A) was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (Cat#9952) from Dr. Warner C. Greene.

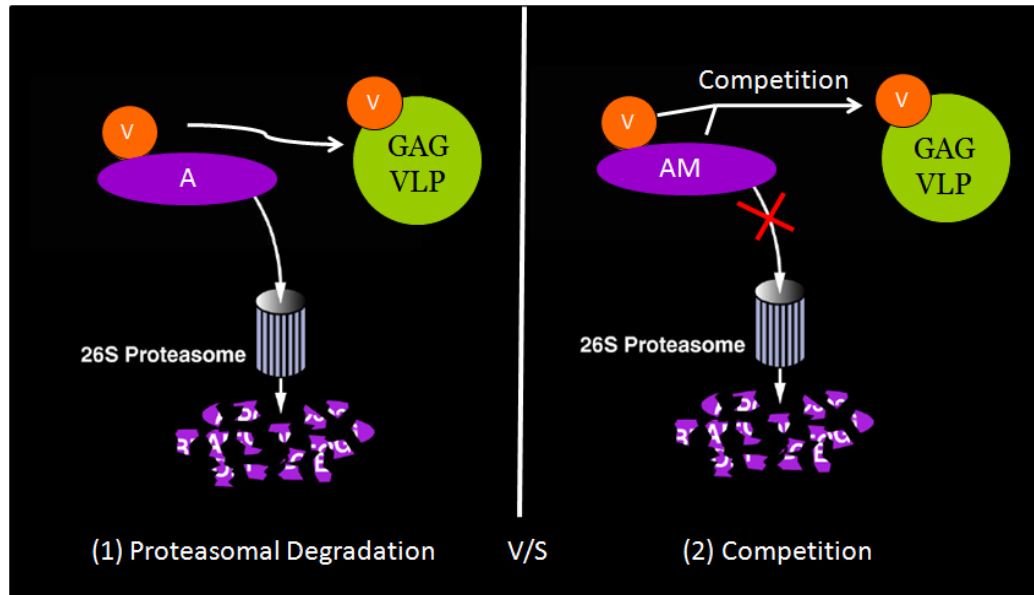


Figure 6.1 Competition hypothesis for the mechanism of Vif action: When both Vif (V) and A3G (A) are co-expressed in the cells along with Pr55^{GAG}, the expressed Vif would deplete the cellular levels of A3G by proteasome-mediated degradation of the latter. Thus the resultant VLPs would only have detectable Vif incorporated into them as shown in (1). If competition was an important mechanism for Vif action, then despite of not being able to degrade the degradation resistant A3G variant (AM), Vif would still be able to compete with it and prevent its incorporation into the budding GAG VLPs as shown in (2).

6.3.3 Construction of the degradation resistant A3G variant

As described in previous reports, replacement of amino acid cysteine at position 97 on the A3G sequence with alanine confers it with resistance to Vif mediated degradation (Opi et al, 2007). The degradation resistant mutant C97A A3G (AM) was constructed by PCR based site-directed mutagenesis of pcDNA3.1-A3G-HA using the forward primer DrC97AF and reverse primer DrC97AR (Table 2.2.1). This produced a mutated A3G in which there had been a substitution of cysteine at position 97 with alanine (Fig. 1.4 Appendix).

6.3.4 pcDNA-HVif (V)

The pcDNA-HVif (V) plasmid was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr. Stephan Bour and Dr. Klaus Strebel. This plasmid expresses a codon-optimized Vif protein of HIV-1 NL4-3 under the control of the CMV promoter.

6.4 Assay

293T cells were plated in 10cm tissue culture dishes and used at 70-80% confluency. Cells were transfected with 10µg each of G and V plasmids together with 10µg of either A or AM plasmid using the standard calcium phosphate transfection protocol (section 2.5.4) (Campanero & Flemington, 1997). As positive controls for the assay, 10µg G plasmid was transfected with 10 µg of either A or AM plasmid in the absence of V. The total amount of plasmid DNA in all dishes was balanced by empty pcDNA3.1 (+) vector. The VLPs obtained from transfected cell supernatants were analysed as described in section 2.8 (Fig. 4.2). Pr55^{GAG} was

probed with mAb to p24, the HA-epitope tagged A3G was probed with monoclonal anti-HA antibody produced in mouse while Vif was detected using the mouse anti-Vif monoclonal antibody (section 2.2.4). The blotted proteins were detected either colorimetrically (section 2.6.3) or by chemiluminescence (section 2.6.4).

6.5 Optimisation of the antibody concentrations to be used

Different concentrations of the above mentioned antibodies were tried to optimise the detection of Vif, A3G and Pr55^{GAG}. In the end optimal concentrations for the detection of these proteins were obtained and these were 1:250 for mouse anti-Vif antibody, 1:10000 for mouse anti-HA antibody and 1:3000 for mAb to p24. A blot obtained from cell extracts expressing the above proteins developed using the optimised antibody concentrations is shown in Fig. 6.2. As the Vif signal with the mouse anti-Vif antibody was very weak, the rabbit HIV-1 HXB2 Vif antiserum was employed for Vif detection in all subsequent experiments.

6.6 Results

293T cells were transfected with either A or AM and G plasmids along with or in the absence of pcDNA-hVif. When extracts from these cells were analysed for the presence of wild type A3G-HA (A), A3G-HA variant (AM) and Pr55^{GAG} (G), there was no visible change in the A3G variant (AM) concentration in cells expressing G, V and AM when compared with cells expressing just G and AM {Fig. 6.3 (1), (2) and (3)}. This was expected as the A3G variant cannot be degraded by Vif. Thus the A3G/GAG ratio in cells transfected with GAG, Vif and AM (GVAM) was similar to those from which Vif was excluded (GAM) {Fig. 6.3 (3)}.

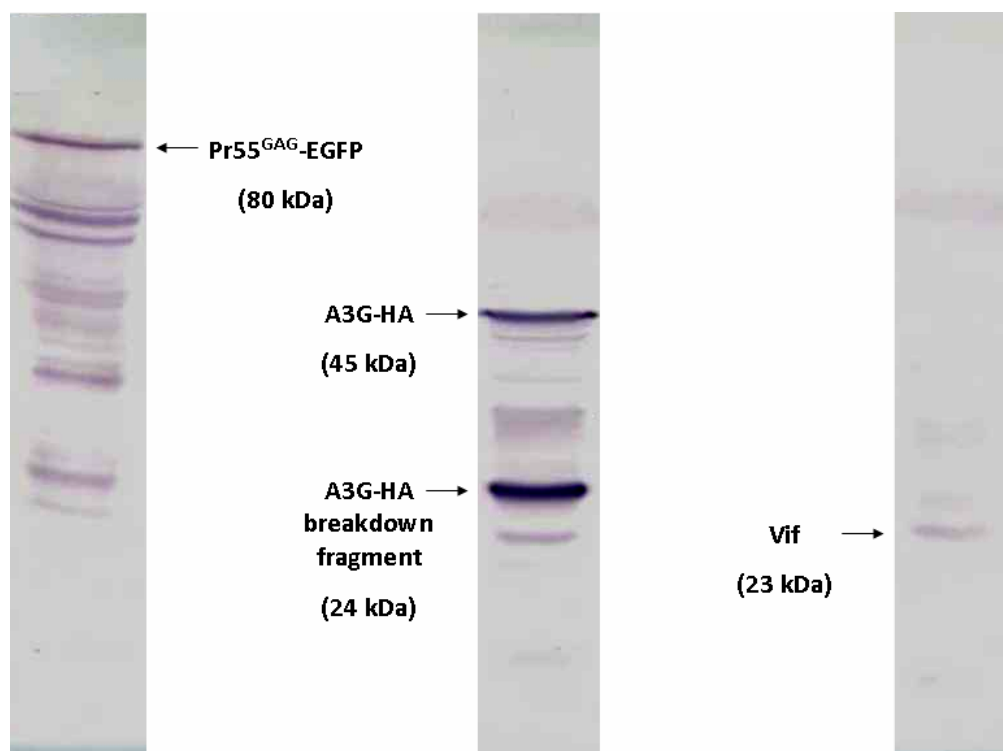


Figure 6.2 Analysis of cell extracts by immunoblotting: 293T cells were transfected with the respective plasmids using the standard calcium phosphate transfection protocol (section 2.5.4). The cell extracts were collected 48 hrs post transfection, electrophoresed on 12.5% SDS-PAGE gel and transferred onto a nitrocellulose membrane (section 2.6). Vif was probed for using a mouse anti-Vif antibody (1:250), A3G-HA using a mouse anti-HA antibody (1: 10,000) and GAG with a mouse anti-p24 antibody (1:3000) described in section 2.2.4. Phosphatase conjugated goat anti-mouse was used as a secondary antibody (1:25,000). The blot was detected colorimetrically using BCIP/NBT alkaline phosphatase substrate tablets (Sigma-Aldrich B5655) dissolved in deionised water (section 2.6.3). The migration positions of Gag-EGFP, A3G-HA & its degradation fragment and Vif are indicated.

However there was a drop observed in the A3G levels in cells expressing G, V and A when compared with cells expressing just G and A {Fig. 6.3 (1), (2) and (3)}. This result was also expected as Vif mediated proteasomal degradation of A3G leads to a decrease in cellular A3G levels. Thus the A3G/GAG ratio in GVA was almost one-third to that observed in GA {Fig. 6.3 (3)}. Fig. 6.3 (4) shows the expression levels of the Vif (V) protein within these cells.

When GAG VLPs obtained from the supernatant derived from transfected cells mentioned above were analysed for the presence of A3G and Pr55^{GAG}, contrary to expectations, the experimental control consisting of VLPs obtained from cells expressing G, V and A showed no visible reduction in the A3G concentration when compared with VLPs obtained from cells expressing just G and A {Fig. 6.4 (1) and (2)}. Densitometric analysis revealed a small decrease in the A3G/GAG ratio of GVA when compared to GA but the magnitude of this reduction was not enough to be accountable for the Vif mediated exclusion of A3G from GAG VLPs {Fig. 6.4 (3)}.

This result was not consistent with previous well-established articles reporting on the mechanism of Vif action. Although a Vif-dependent decrease in A3G levels in cell extracts was observed, however a corresponding decrease in A3G levels from the VLPs derived from these cells was not seen. This meant that the positive control for the experiment was not functioning as expected, the reasons for which are discussed in the next section.

Figure 6.3 Analysis of extracts derived from cells expressing either A3G or the A3G variant and Pr55^{GAG} in the presence or absence of Vif: 293T cells were transfected with the indicated plasmids using the standard calcium phosphate transfection protocol (section 2.5.4). Cell extracts were collected 48 hrs post transfection, electrophoresed on 12.5% SDS-PAGE gel and transferred onto a nitrocellulose membrane and analysed by immunoblotting as described in Materials and Methods.

(1) A3G-HA was detected by the mouse anti-HA antibody (1: 10000) and

(2) GAG was detected by the mouse anti-p24 antibody (1:3000).

Peroxidase conjugated goat anti-mouse (1:5000) was used as the secondary antibody.

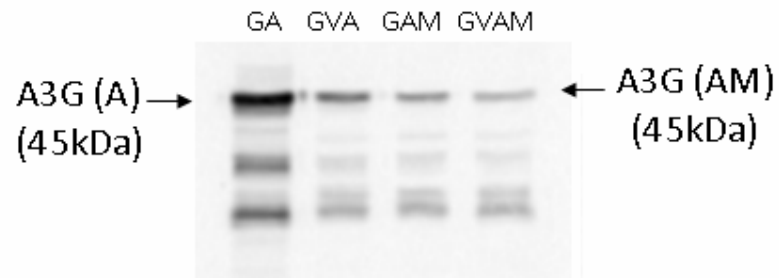
(3) Computer-aided densitometric analysis of A3G and GAG protein bands in (1) &

(2). The bar chart depicts A3G/GAG ratio against the combination of plasmids transfected.

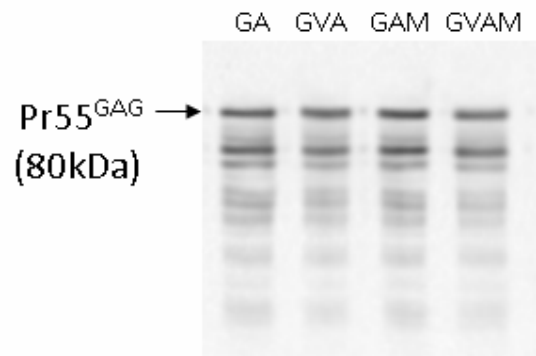
(4) Analysis of the expression levels of Vif in cell extracts mentioned above using a rabbit Vif antiserum (1:1000) as the primary antibody. Peroxidase conjugated goat anti-rabbit (1:100,000) was used as the secondary antibody.

All the proteins were detected by using an ECL Chemiluminescence detection kit (GE Healthcare Life Sciences) and analysed using the Bio-Rad ChemiDoc XRS imaging system. The track labelling indicates the respective plasmids transfected. The positions of Pr55^{GAG} (G), A3G (A), the degradation resistant A3G Variant (AM) and Vif (V) are arrowed.

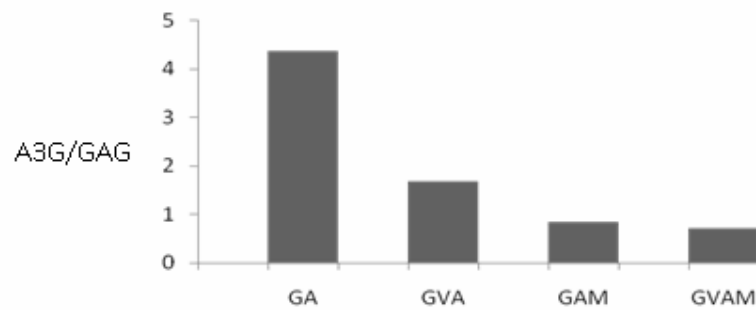
(1)



(2)



(3)



(4)

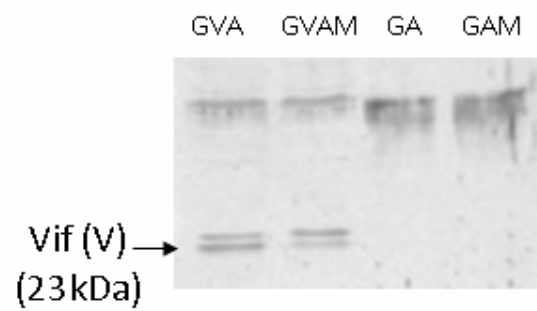


Figure 6.4 Analysing the amount of A3G protein incorporated in GAG VLPs in the presence or absence of Vif:

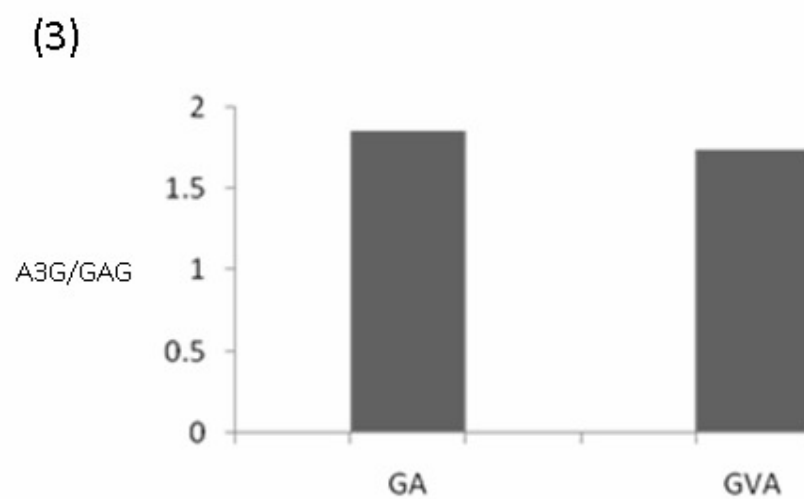
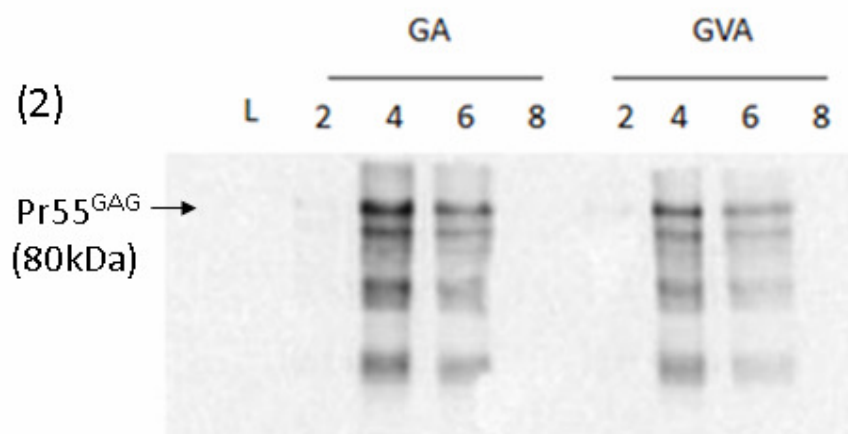
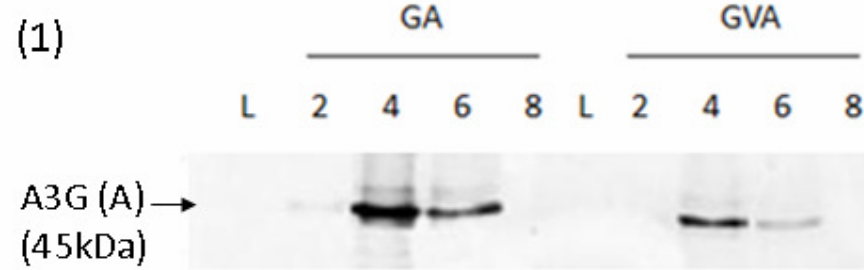
GAG VLPs harvested from supernatant of 293T cells expressing (i) Pr55^{GAG} & A3G (GA) and (ii) Pr55^{GAG}, Vif & A3G (GVA) were fractionated on 20-60% sucrose gradients and gradient fractions analysed by SDS-PAGE and western blotting as described in Materials and Methods.

(1) A3G-HA was detected by using mouse anti-HA antibody (1:10,000)

(2) GAG was detected by using mouse anti-p24 antibody (1:3000)

Peroxidase conjugated goat anti-mouse (1:5000) was used as the secondary antibody in all blots. Proteins were detected using an ECL-Chemiluminescence detection kit (GE Healthcare Life Sciences) and analysed using the Bio-Rad ChemiDoc XRS imaging system as described in Materials and Methods. The numbering of the gel tracks denotes the specific sucrose gradient fraction analysed while (L) denotes the position of the Ladder. The positions of Pr55^{GAG} and A3G are arrowed.

(3) Computer-aided densitometric analysis of A3G and GAG protein bands in (1) and (2). The bar chart depicts the A3G/GAG ratio in the GAG VLPs.



When VLPs derived from cells transfected with G and AM in the presence or absence of V were analysed for the levels of the A3G variant and Pr55^{GAG}, no visible decrease in the amount of A3G variant was observed in VLPs derived from cells expressing G, V and AM when compared to VLPs obtained from cells expressing only G and AM {Fig. 6.5 (1) and (2)}. Thus the additional expression of Vif did not have any effect on the concentration of AM in virus-like particles. Densitometric analysis of the protein bands revealed a small decrease in the A3G/GAG ratio of GVAM when compared with GAM but the magnitude of this reduction was not comparable to what could be expected in the scenario of Vif mediated exclusion of A3G from GAG VLPs {Fig. 6.5 (3)}.

6.7 Summary

Although a 3-fold decrease in A3G concentration was detected in the cell extracts transfected with A3G, Pr55^{GAG} and Vif plasmids when compared to the cells transfected with just A3G and Pr55^{GAG}, a similar fall in the levels of A3G was not reflected in VLPs derived from those cells {Fig. 6.3 & Fig. 6.4 }. It was expected that due to the proteasome-mediated degradation of A3G by Vif, the levels of A3G in the cells would reduce leading to a corresponding decrease in A3G levels in the GAG VLPs obtained from those cells (Yu et al, 2003). But this was not found to be the case with the A3G/GAG ratio in VLPs with GVA being similar to that of GA {Fig. 6.4 (3)}. Thus the positive control for the experiment itself was not functioning optimally.

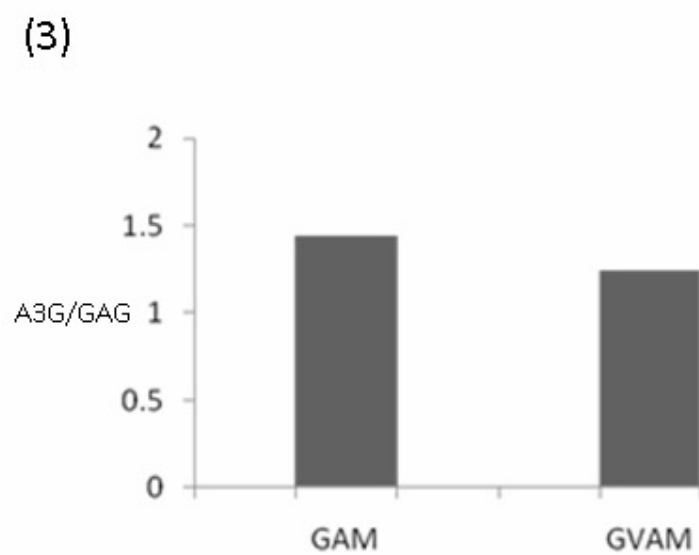
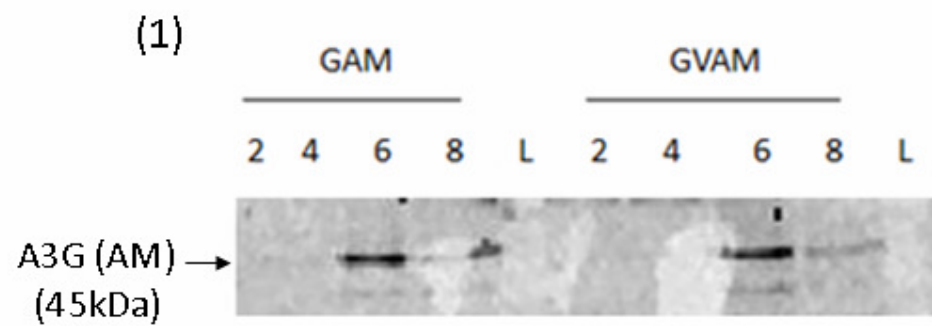
Figure 6.5 Comparison of the amounts of the degradation resistant A3G variant getting incorporated into GAG VLPs in the presence or absence of Vif: GAG VLPs harvested from supernatant of 293T cells expressing (i) Pr55^{GAG} & degradation-resistant A3G variant (GAM) and (ii) Pr55^{GAG}, Vif and degradation-resistant A3G (GVAM) were fractionated on 20-60% sucrose gradients and gradient fractions analysed by SDS-PAGE and western blotting as described in Materials and Methods.

(1) A3G-HA variant (AM) was detected by using a mouse anti-HA antibody (1:10,000)

(2) GAG was detected by using a mouse anti-p24 antibody (1:3000)

Peroxidase conjugated goat anti-mouse (1:5000) was used as the secondary antibody in all blots. Proteins were detected using an ECL-Chemiluminescence detection kit (GE Healthcare Life Sciences) and analysed using the Bio-Rad ChemiDoc XRS imaging system as described in Materials and Methods. The numbering of the gel tracks denotes the specific sucrose gradient fraction analysed while (L) denotes the position of the ladder. The positions of Pr55^{GAG} and the A3G variant are arrowed.

(3) Computer-aided densitometric analysis of A3G and GAG protein bands in (1) and (2). The bar chart depicts the A3G/GAG ratio in the GAG VLPs.



One reason for this could be the non-specific incorporation of A3G in GAG VLPs. As during the experiment, all the proteins were over-expressed; this could lead to increased levels of A3G which could then non-specifically interact with Pr55^{GAG} to get incorporated into the budding GAG VLPs.

In light of the observations obtained from the positive control, it becomes very difficult to analyse the results of the experiment itself. Using the A3G variant (AM) in place of wild type A3G resulted in no change in the variant levels in GAG VLPs in the presence/absence of Vif {Fig. 6.5 (1), (2) and (3)}.

In the cell extracts transfected with Pr55^{GAG}, Vif and the A3G variant, there was no significant change in the variant levels either in the presence or in the absence of Vif. It is interesting to note here that the steady state levels of the degradation resistant mutant seen in cell extracts were almost 3-fold lower than those of wild-type A3G {Fig. 6.3 (1) and (3)}. This could either be due to the variant being constitutively unstable and thus having a lower half-life than wild type A3G or due to the expression levels of the variant from the plasmid being lower than the wild type A3G. The latter seems less likely as both the wild type as well as the degradation resistant mutant were being transcribed under the control of the same promoter (CMV).

Chapter 7

Analysis of interactions of Vif and A3G with Pr55^{GAG} in a VLP assembly assay

7.1 Background:

By employing the mammalian two-hybrid assay, Vif and A3G were both shown to interact on the same site with Pr55^{GAG} (Chapter 3). To obtain independent evidence in support of these interactions, it was decided to study them using the GAG VLP assembly assay. The sucrose gradient assay protocol has been described in section 2.8.

7.2 Plasmids used in the assay

The pcDNA-HVif plasmid has been described in section 6.3.4. A 3×HA tagged version of pcDNA-HVif was constructed for use in this assay. The triple HA tags in pcDNA3.1-A3G-HA were amplified by PCR using forward primer EcoRIHAF and reverse primer EcoRIHAR {Table 2.2.1 and Fig. 7.1 (1)&(2)}. The amplified PCR product and vector pcDNA-HVif were both digested with restriction enzyme EcoRI so that the insert can be ligated in frame with the Vif gene sequence of the vector resulting in the formation of HA-pcDNA-HVif (*V) {Fig. 7.1 (3) & Fig. 1.5 Appendix}. The correct orientation of the HA tag into the Vif expression plasmid was first verified by restriction digestion of the mutant with the enzyme NdeI and subsequent sequencing.

In addition all the other plasmids used in this assay namely the Rev-independent GAG expression construct (G), the basic linker mutant of Pr55^{GAG} (GM), and

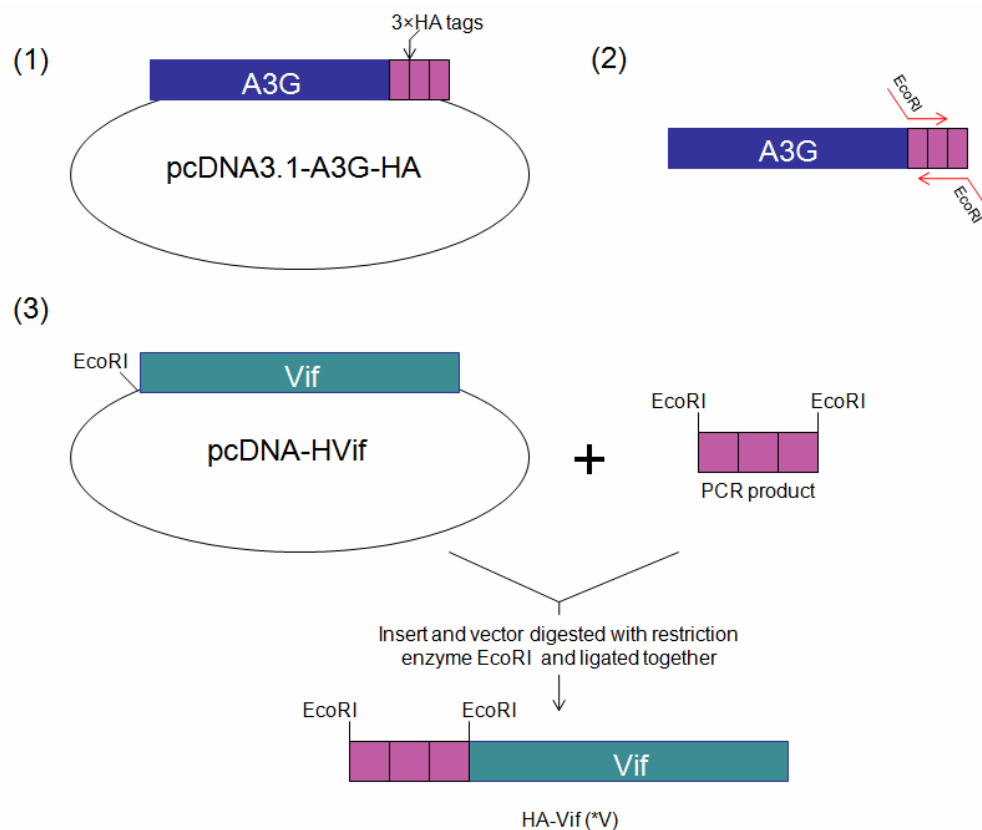


Figure 7.1 Schematic of construction of the 3xHA tagged version of Vif (*V):

(1) pcDNA3.1-A3G-HA: Schematic diagram showing the 3xHA tagged version of A3G in pcDNA3.1 vector (2) Amplification of the 3xHA tags by PCR: The triple HA tags at the C-terminal end of A3G sequence in pcDNA3.1-A3G-HA vector were amplified by PCR. The PCR primers were flanked by EcoRI restriction enzyme sites for subsequent restriction digestion of the PCR product (3) Construction of N-terminal 3xHA tagged version of Vif: The amplified PCR product (insert) and pcDNA-HVif (vector) were treated with the restriction enzyme EcoRI. The digested insert and vector were ligated together to construct an N-terminally HA-tagged version of Vif such that the HA tags were in the same frame as the Vif sequence. The correct orientation of the HA tags was verified first by restriction digestion of the resultant plasmid with enzyme NdeI and subsequent sequencing (Fig. 1.5 Appendix).

pcDNA-3.1-A3G-HA (A) have been already described in sections 4.2, 4.3 and 6.3.2 respectively.

7.3 Assay protocol

The assay protocol for the sucrose gradient analysis of GAG VLPs has been detailed in section 2.8 of the Materials and Methods. 293T cells were transfected using the calcium phosphate transfection protocol (section 2.5.4) with 15µg of each plasmid in the following combinations:

(i) *V and G

(ii) *V and GM

(iii) A and G

(iv) A and GM

The GAG VLPs in the resultant cell culture supernatants were overlaid onto linear 20-60% sucrose gradients and subjected to centrifugation in a Beckman SW40Ti rotor for 16 hrs at 4°C. After ultracentrifugation, equal fractions were collected from the bottom of the gradient, fractionated through 12.5% SDS-PAGE and analysed by immunoblotting (section 2.6).

7.4 Hypothesis

A significant reduction in the incorporation of both V and A in the mutant GAG VLPs in comparison to wild-type GAG VLPs would signify that neither Vif nor A3G interacts with the basic linker mutant of Pr55^{GAG}. This would in turn be a proof that the basic linker region of the nucleocapsid domain is important for the interaction of both these proteins with Pr55^{GAG} implying they interact at the same site on Pr55^{GAG}.

7.5 Results

When VLPs obtained from 293T cells transfected with *V and G were analysed for the presence of Vif and Pr55^{GAG}, both Vif and GAG were found to be concentrated in the same fraction of the gradient (aliquot number 6). Similarly in VLPs obtained from cells where Vif was co-expressed with GM, no visible difference was detected in the amount of Vif getting incorporated into the mutant GAG VLPs when compared to wild type GAG VLPs. Densitometric analysis of Vif and the wild type & mutant Pr55^{GAG} bands revealed a small reduction in the amount of HA-Vif in VLPs obtained from cells co-transfected with *V and GM when compared with VLPs obtained from *V and G however this decrease was not comparable to the huge drop observed (~40 fold) in CAT reporter expression in the two-hybrid assay driven by AD-Vif and BD-NCSUB (Fig. 7.2 and Figs. 3.11 and 3.12).

When 293T cells were transfected with either G or GM plasmids and pcDNA-3.1-A3G-HA, the highest concentration of both A3G and Pr55^{GAG} was detected in the same aliquot of the gradient (aliquot 4). No visible difference was observed in the levels of A3G in mutant GAG VLPs when compared to wild type GAG VLPs. On densitometric analysis of the protein bands obtained, a small reduction in the amount of A3G was observed in the VLPs obtained from cells co-transfected with A and GM when compared to the VLPs obtained from A and G however this reduction was not comparable to the significant drop (~40 fold) in CAT reporter expression seen in the two-hybrid assay driven by AD-A3G and BD-NCSUB (Fig. 7.3 and Figs. 3.8 and 3.9).

Figure 7.2 Application of the VLP assembly assay for studying the interactions of HA-Vif with Pr55^{GAG}: GAG VLPs harvested from the supernatant of 293T cells expressing (i) Pr55^{GAG} & HA-Vif (*VG), and (ii) Basic linker GAG mutant & HA-Vif (*VGM) were fractionated on 20-60% sucrose gradients and gradient fractions analysed by SDS-PAGE and western blotting as described in Materials and Methods.

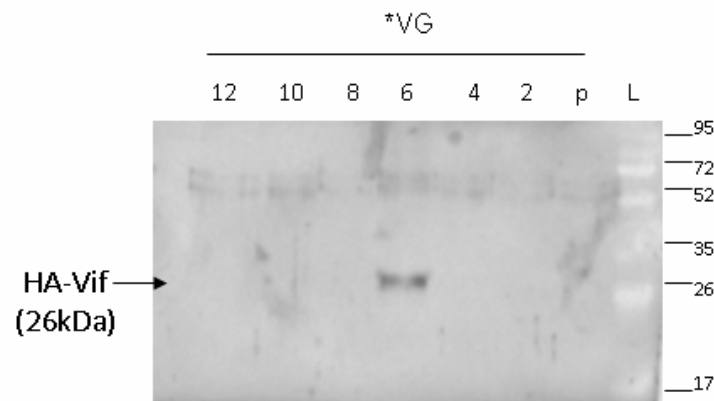
(1 & 2) HA-Vif was detected by using mouse anti-HA antibody (1:10,000).

(3) GAG was detected by using mouse anti-p24 antibody (1:3000).

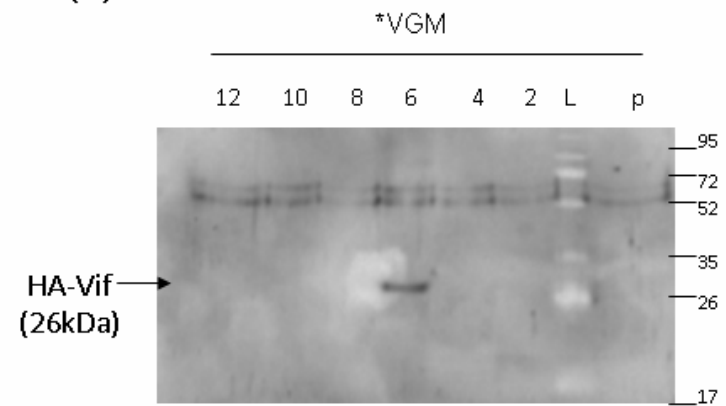
Peroxidase conjugated goat anti-mouse (1:5000) was used as the secondary antibody in all blots. Proteins were detected using an ECL-Chemiluminescence detection kit (GE Healthcare Life Sciences) and analysed using the Bio-Rad ChemiDoc XRS imaging system as described in Materials and Methods. The numbering of the gel tracks denotes the specific sucrose gradient fraction analysed while (L) denotes the position of the ladder. The positions of Pr55^{GAG}, the GAG mutant and HA-Vif are arrowed.

(4) Computer-aided densitometric analysis of HA-Vif and GAG protein bands in (1), (2) and (3). The bar chart depicts the Vif/GAG ratio in GAG VLPs.

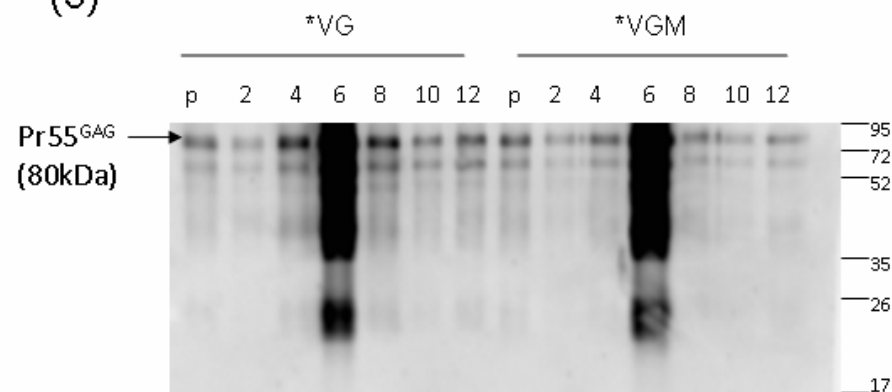
(1)



(2)



(3)



(4)

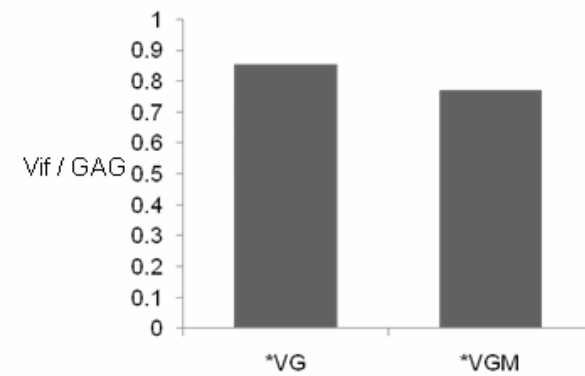


Figure 7.3 Application of the VLP assembly assay for studying the interactions of A3G with Pr55^{GAG}: GAG VLPs harvested from the supernatant of 293T cells expressing (i) Pr55^{GAG} & A3G (AG), and (ii) Basic linker GAG mutant & A3G (AGM) were fractionated on 20-60% sucrose gradients and gradient fractions analysed by SDS-PAGE and western blotting as described in Materials and Methods.

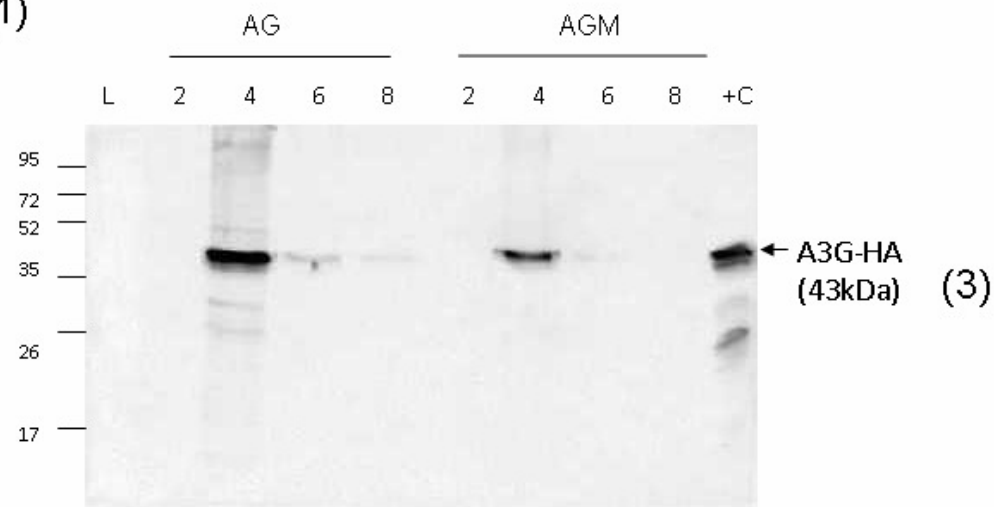
(1) A3G-HA was detected by using mouse anti-HA antibody (1:10,000). Peroxidase conjugated goat anti-mouse (1:5000) was used as the secondary antibody. A3G was detected using an ECL-Chemiluminescence detection kit (GE Healthcare Life Sciences) and analysed using the Bio-Rad ChemiDoc XRS Imaging System as described in Materials and Methods.

(2) The antibodies in (1) were stripped off the blot by using Stripping Buffer (section 2.6.5) and the same blot was reprobed with anti-p24 antibody (1:3000) for the detection of GAG. Alkaline Phosphatase conjugated goat anti-mouse (1:25000) was used as the secondary antibody. The blot was detected colorimetrically using BCIP/NBT alkaline phosphatase substrate tablets (Sigma-Aldrich B5655) dissolved in deionised water (section 2.6.3).

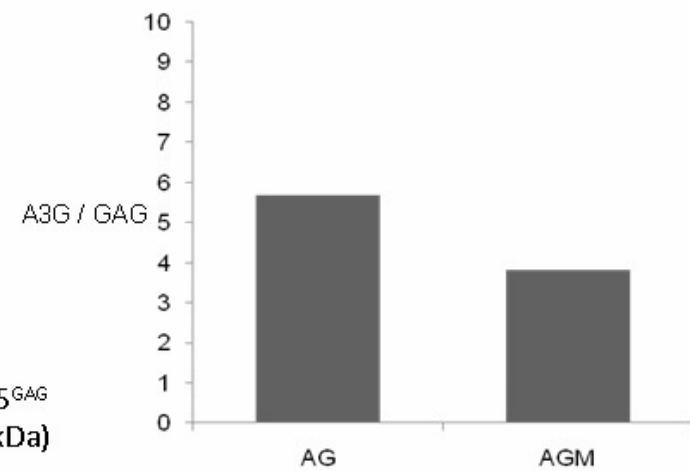
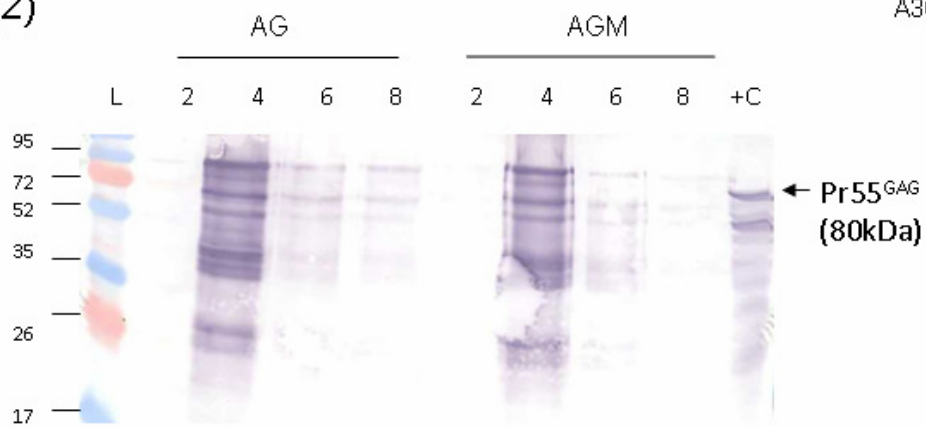
The numbering of the gel tracks denotes the specific sucrose gradient fraction analysed while (L) denotes the position of the Ladder. The positions of Pr55^{GAG}, the GAG mutant and A3G are arrowed. Positive control (+C) is cell extracts transfected with A3G and Pr55^{GAG} (AG).

(4) Computer-aided densitometric analysis of A3G and GAG protein bands in (1) and (2). The bar chart depicts the A3G/GAG ratio in GAG VLPs.

(1)



(2)



Densitometric analysis of the bands revealed that the reduction in the A3G levels observed in GM VLPs was two-fold more than the reduction in the Vif levels {Fig. 7.2 (4) & 7.3 (3)}.

7.6 Summary

A significant drop in the levels of both Vif and A3G in the VLPs derived from the basic linker mutant of Pr55^{GAG} was expected. In the mammalian two-hybrid studies described in Chapter 3, a 40-fold reduction in the CAT reporter expression levels was detected with the basic linker mutant when compared with wild type Pr55^{GAG} suggesting that the mutation in this region had a very critical impact on the interactions of both these proteins with Pr55^{GAG}. However, in the VLP assembly assay, a relatively small decrease was observed in the levels of both Vif and A3G in the VLPs obtained from cells transfected with the basic linker GAG mutant when compared with VLPs obtained from cells expressing wild-type Pr55^{GAG}.

This result is difficult to interpret when analysed in conjunction with previous observations detailed in Chapter 6. As discussed in section 6.7, non-specific incorporation of A3G was observed in the VLPs when A3G and Pr55^{GAG} were allowed to co-express in cells.

The experimental set-up for this assay was essentially the same as that used in Chapter 6. Thus it is possible that the assay is confounded by non-specific incorporation of these proteins, obviating any possibility of detecting changes in their specific incorporation into assembling virus-like particles.

Also of note here is the VLPs being concentrated in different aliquot number of the sucrose gradient in case of Vif and A3G. While Vif and Pr55^{GAG} were found to be concentrated in aliquot number 6, A3G and Pr55^{GAG} were detected in aliquot number 4. This difference is possibly due to the method involved in preparing the sucrose gradient and the instrument error introduced while preparing it. As only a maximum of two gradients could be prepared at a time, to obviate this error, the samples to be compared (eg VG and VGM or AG and AGM) were prepared at the same time so that the gradients in their case were the same.

CHAPTER 8

Co-localisation studies of Vif and A3G with

Pr55^{GAG}

8.1 Introduction

Having shown that both Vif and A3G interact with Pr55^{GAG} in the mammalian two-hybrid assay set-up and subsequently having detected their presence in GAG VLPs, it was of great interest to study their localisation inside cells and the dynamics of their positions within cells relative to each other. It was also important to study whether the mutations in Pr55^{GAG} which affected binding with Vif and A3G resulted in a change in their localisation profiles with respect to Pr55^{GAG} or not. These questions were addressed by immunofluorescence imaging of cells expressing Vif/A3G and wild-type Pr55^{GAG}/MutGAG using confocal microscope.

8.2 Background

A number of studies have been reported on the localisation of Vif inside cells. While some reports suggest that it localises to the cytoplasmic membrane as well as being diffusely distributed throughout the cytoplasm (Goncalves *et al.*, 1994; Simon *et al.*, 1997), others have suggested that Vif localises to the cytoskeleton and that this localisation is dependent upon intermediate filament vimentin (Karczewski & Strebel, 1996). Goncalves *et al.* and Karczewski *et al.* also reported observing a light nuclear staining for Vif in their studies in addition to the dominant cytosolic form with the precise amount varying from preparation to preparation. Karczewski *et al.* also observed the nuclear localisation of Vif in cells lacking vimentin (Karczewski & Strebel, 1996). The difference seen in localisation patterns can be attributed to differences in experimental protocol and cell lines used. Goncalves *et al.* used antibody staining of transiently transfected COS cells (Goncalves *et al.*, 1994), whereas Karczewski *et al.* performed their imaging studies in transfected HeLa cells

(Karczewski & Strebel, 1996) and Simon *et al.* used HIV-1 infected H9 cells (Goncalves et al, 1994; Simon et al, 1997).

In contrast to Vif, the position of Pr55^{GAG} in cells is much more constant. It has been reported to localise at the cytoplasmic membrane to initiate particle assembly (Hermida-Matsumoto & Resh, 2000). In fact Simon *et al.* have already reported that Vif and GAG co-localise at the plasma membrane in HIV-1 infected cells (Simon et al, 1997).

In case of A3G, one group has shown A3G is distributed throughout the cytoplasm, with particular concentration occurring at punctuate cytoplasmic collections known as P bodies (Wichroski et al, 2005; Wichroski et al, 2006). However Burnett *et al.* were unable to observe the P body localisation of A3G, but rather reported co-localisation of A3G with Pr55^{GAG} at the plasma membrane and postulated that Gag was responsible for the membrane recruitment of A3G (Burnett & Spearman, 2007).

8.3 Plasmids and antibodies used in the assay

The rev-independent GFP tagged GAG expression plasmid pGAG-EGFP (G) and mutant plasmid MutGAG (GM) incorporating the basic linker mutations abrogating Vif and A3G interaction with Pr55^{GAG} have already been described in sections 4.2 and 4.3. The HA-tagged A3G expressing plasmid pcDNA-3.1-A3G-HA has been described in section 6.3.2 while the codon-optimised Vif expression plasmid pcDNA-hVif has been described in section 6.3.4.

The GFP-tagged G and GM proteins were visualised by detecting GFP fluorescence. Vif was detected using antibody staining with a rabbit HIV-1_{HXB2} Vif antiserum (1:200 dilution) and HA-tagged A3G was detected using the mouse anti-HA

antibody (Sigma-Aldrich Cat. No. H9658 used at 1:250 dilution). Red fluorescent Alexa Fluor 594 conjugated goat anti-rabbit (Invitrogen Cat. No. A-11012 at 1:3000 dilution) and goat anti-mouse (Invitrogen Cat. No. A-11005 at 1:500 dilution) antibodies were used as secondary antibodies.

8.4 Assay

293 T cells were used for the immunofluorescence studies described in this chapter. Cells were transfected with the following combinations of plasmids:

- | | |
|-----------------------------------|---------------------|
| (i) Vif and Pr55 ^{GAG} | (ii) Vif and MutGAG |
| (iii) A3G and Pr55 ^{GAG} | (iv) A3G and MutGAG |

The controls for the assay were the following plasmids transfected individually:

- | | |
|---------------------------|-------------|
| (i) Vif | (ii) A3G |
| (iii) Pr55 ^{GAG} | (iv) MutGAG |

Post-transfection the cells were processed as described in section 2.9 and observed under the Leica sp5 confocal microscope.

8.5 Results

When 293T cells were co-transfected with Vif and wild-type GAG plasmids, the signal for wild-type Pr55^{GAG} (G) was more intense at the cell membrane where it accumulates to assemble into virus particles. A diffuse signal was also seen throughout the cell (Fig. 8.1 B). The red fluorescent Vif showed diffuse distribution throughout the cytoplasm (Fig. 8.1 C). There was a more limited Vif fluorescence also observed in the nucleus overlapping the blue signal from nuclear stain DAPI (Fig. 8.1 C and D). The signal for Vif was much weaker than that for the G-GFP fusion protein. Although a subset of the expressed Vif protein was seen to be

overlapping with G at the cytoplasmic membrane, it was not possible to definitively conclude from these data that Vif is localising with Pr55^{GAG} at the membrane.

When 293T cells were co-transfected together with plasmids expressing Vif and mutant Pr55^{GAG}, no visible change was seen in cellular localisation of either Vif or the Pr55^{GAG} mutant (GM) compared to that seen with G. The signal for GM was stronger at the cytoplasmic membrane (Fig. 8.2 B) but some diffuse signal for the protein was seen throughout the cytoplasm. The Vif signal was also spread diffusely across the cytoplasm but there was some overlap with the blue DAPI signal in the nucleus (Fig. 8.2 C and D). A fraction of Vif did overlap with GM at the plasma membrane but again this evidence was not sufficient to suggest a colocalisation between Vif and GM.

Thus no visible change was observed in the cellular localisation profiles of Vif and the Pr55^{GAG} mutant compared to that seen when Vif and wild type Pr55^{GAG} were co-expressed in cells (Fig. 8.1 A and 8.2 A).

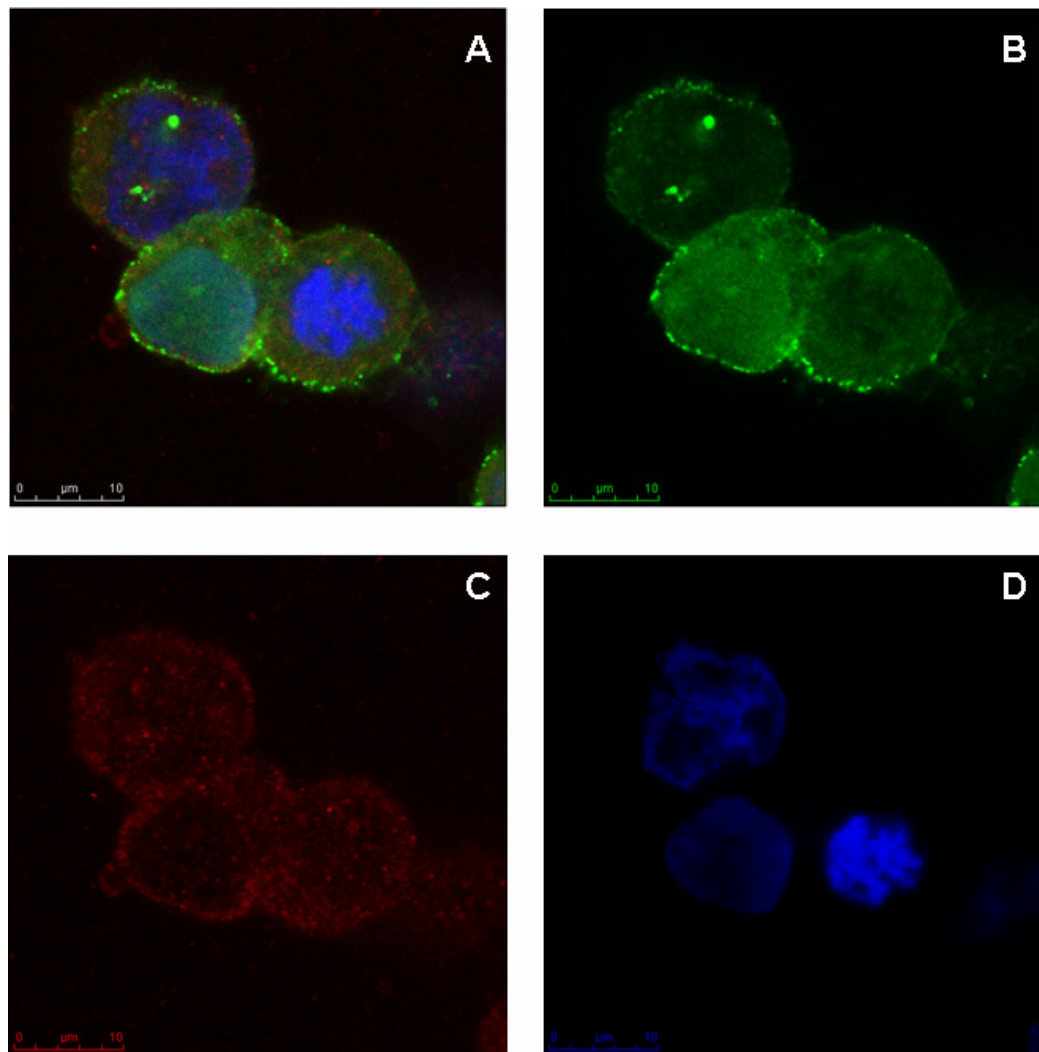


Figure 8.1 Localisation of co-expressed Vif and wild-type Pr55^{GAG} (G) proteins: 293T cells were co-transfected with plasmids expressing Pr55^{GAG}-GFP fusion and Vif proteins using the standard lipofectamine transfection protocol detailed in section 2.5.3. 48 hours post-transfection, the cells were processed and analysed as described in section 2.9. GFP fluorescence indicates the position of the GAG-GFP fusion proteins. Vif was detected by using a Rabbit HIV-1_{HXB2} Vif antiserum as primary antibody followed by red fluorescent Alexa Fluor 594 goat anti-rabbit as the secondary antibody. Nuclei are stained blue with the nuclear stain DAPI. Panel B shows the position of the GAG-GFP fusion protein. Panel C depicts the position of the red fluorescent Vif protein and panel D shows the nuclei of cells stained with DAPI. Panel A image is the merge of images B, C and D. A magnification bar of 10 μm is present in the bottom left corner of each image.

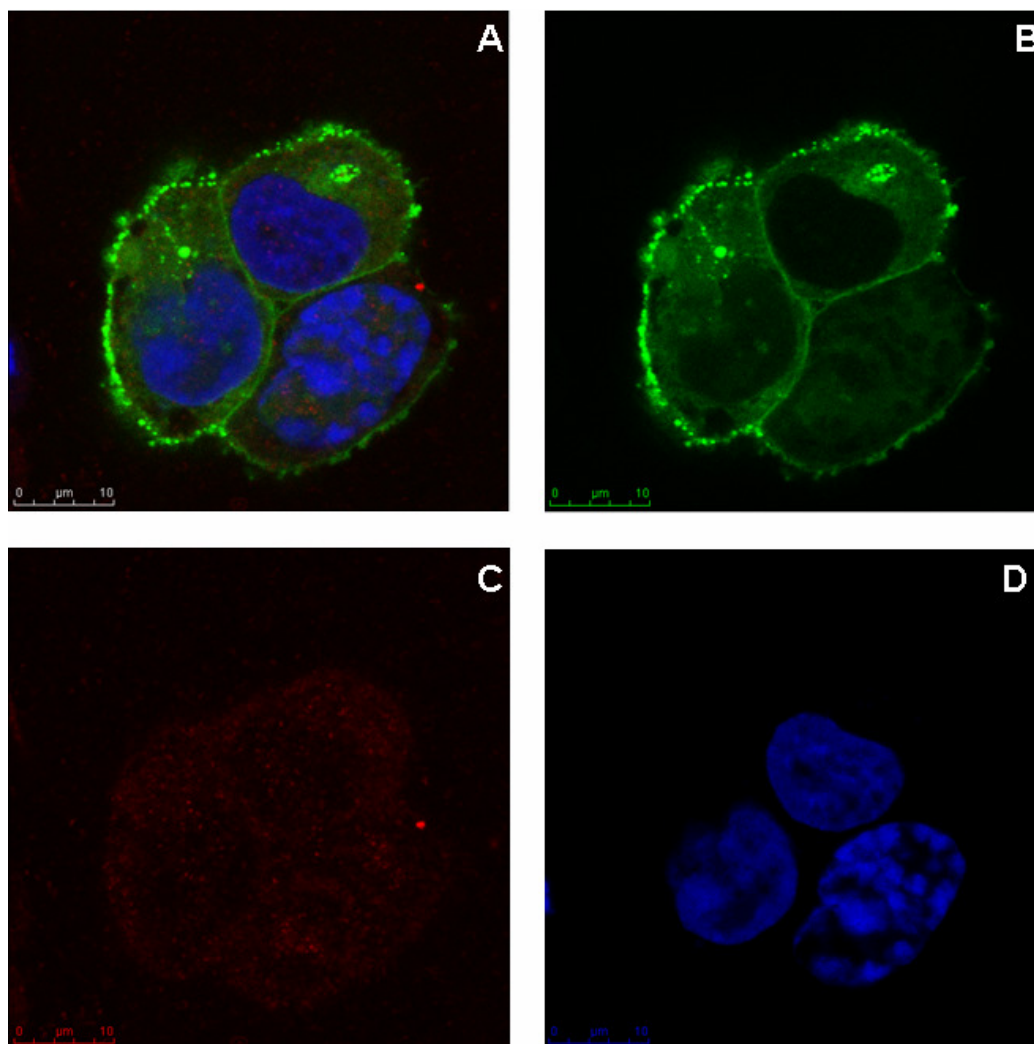


Figure 8.2 Localisation of co-expressed Vif and basic linker mutant Pr55^{GAG} (GM) proteins: 293T cells were co-transfected with plasmids expressing the mutant Pr55^{GAG}-GFP fusion and Vif proteins using the standard lipofectamine transfection protocol detailed in section 2.5.3. 48 hours post-transfection, the cells were processed and analysed as described in section 2.9. GFP fluorescence indicates the position of the mutant GAG. Vif was detected by using a Rabbit HIV-1 HXB2 Vif antiserum as primary antibody followed by red fluorescent Alexa Fluor 594 goat anti-rabbit as the secondary antibody. Nuclei are stained blue with the nuclear stain DAPI. Panel B shows the position of the GAG mutant in cells. Panel C depicts the position of the red fluorescent Vif protein and panel D shows the nuclei of cells stained with DAPI. Panel A image is the merge of images B, C and D. A magnification bar of 10 μm is present in the bottom left corner of each image.

When the HA-tagged A3G and wild-type Pr55^{GAG} – GFP fusion proteins were co-expressed in 293T cells, Pr55^{GAG} was again seen to concentrate at the cytoplasmic membrane, a diffuse GAG signal was also detected throughout the cell (Fig. 8.3 B).

By contrast the HA-tagged A3G, localised primarily to the cytoplasmic compartment with a subset of the protein co-localising with Pr55^{GAG} at the plasma membrane (Fig. 8.3 A and C). Also in contrast to Vif which was visibly localised both in the cytoplasm and the nucleus, A3G was exclusively observed in the cytoplasm of cells (8.1 C, D and 8.3 C, D).

When HA-tagged A3G was co-expressed with the basic linker mutant of Pr55^{GAG}, again no visible change was observed in its localisation compared to that seen when co-expressed with wild type Pr55^{GAG} (Fig. 8.3 and 8.4).

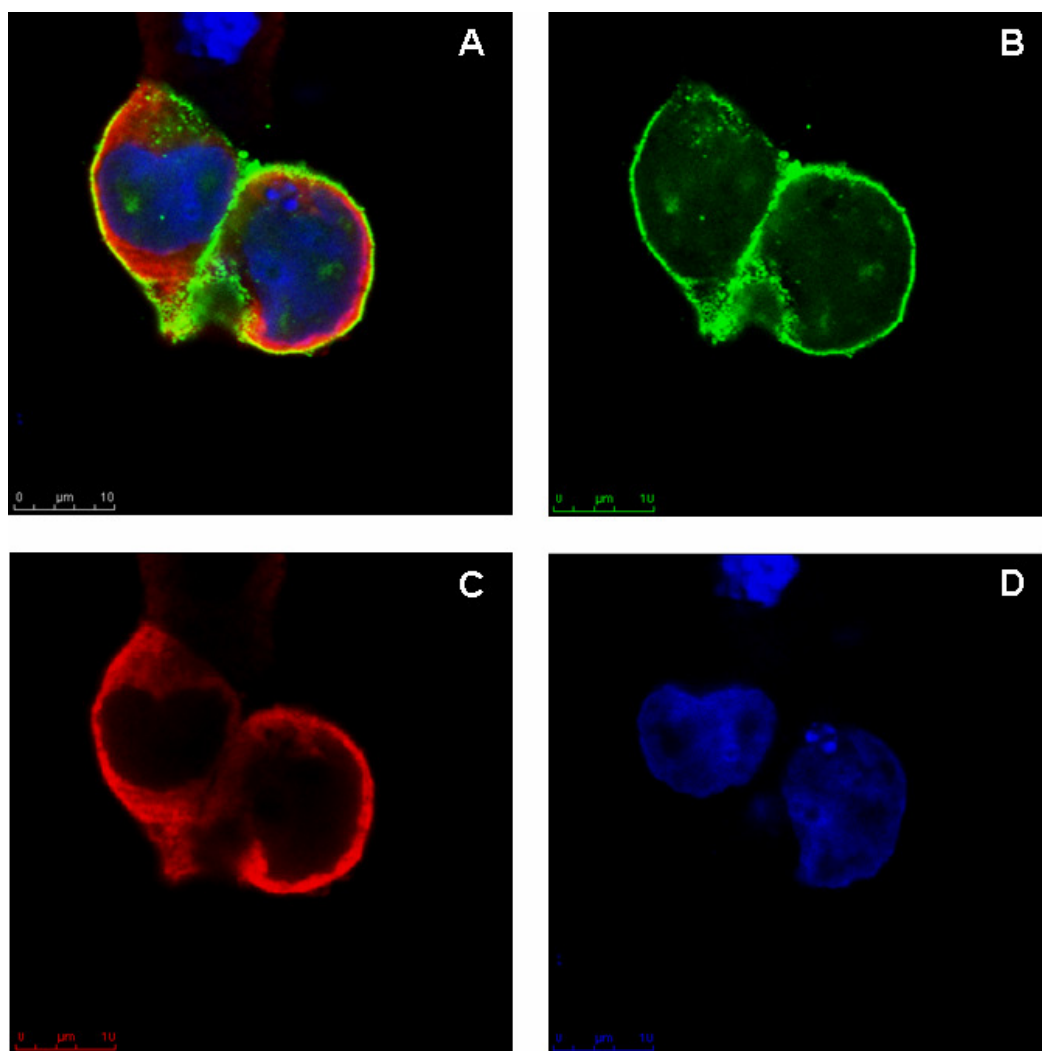


Figure 8.3 Localisation of co-expressed A3G and wild-type Pr55^{GAG} (G) proteins: 293T cells were co-transfected with plasmids expressing Pr55^{GAG}-GFP fusion and HA-tagged A3G proteins using the standard lipofectamine transfection protocol detailed in section 2.5.3. 48 hours post-transfection, the cells were processed and analysed as described in section 2.9. GFP fluorescence indicates the position of the GAG-GFP fusion proteins. A3G was detected by using mouse anti-HA as the primary antibody followed by red fluorescent Alexa Fluor 594 goat anti-mouse as the secondary antibody. Nuclei are stained blue with the nuclear stain DAPI. Panel B shows the position of the GAG-GFP fusion protein. Panel C depicts the position of the red fluorescent HA-tagged A3G and panel D shows the nuclei of cells stained with DAPI. Panel A image is the merge of images B, C and D. A magnification bar of 10 μm is present in the bottom left corner of each image.

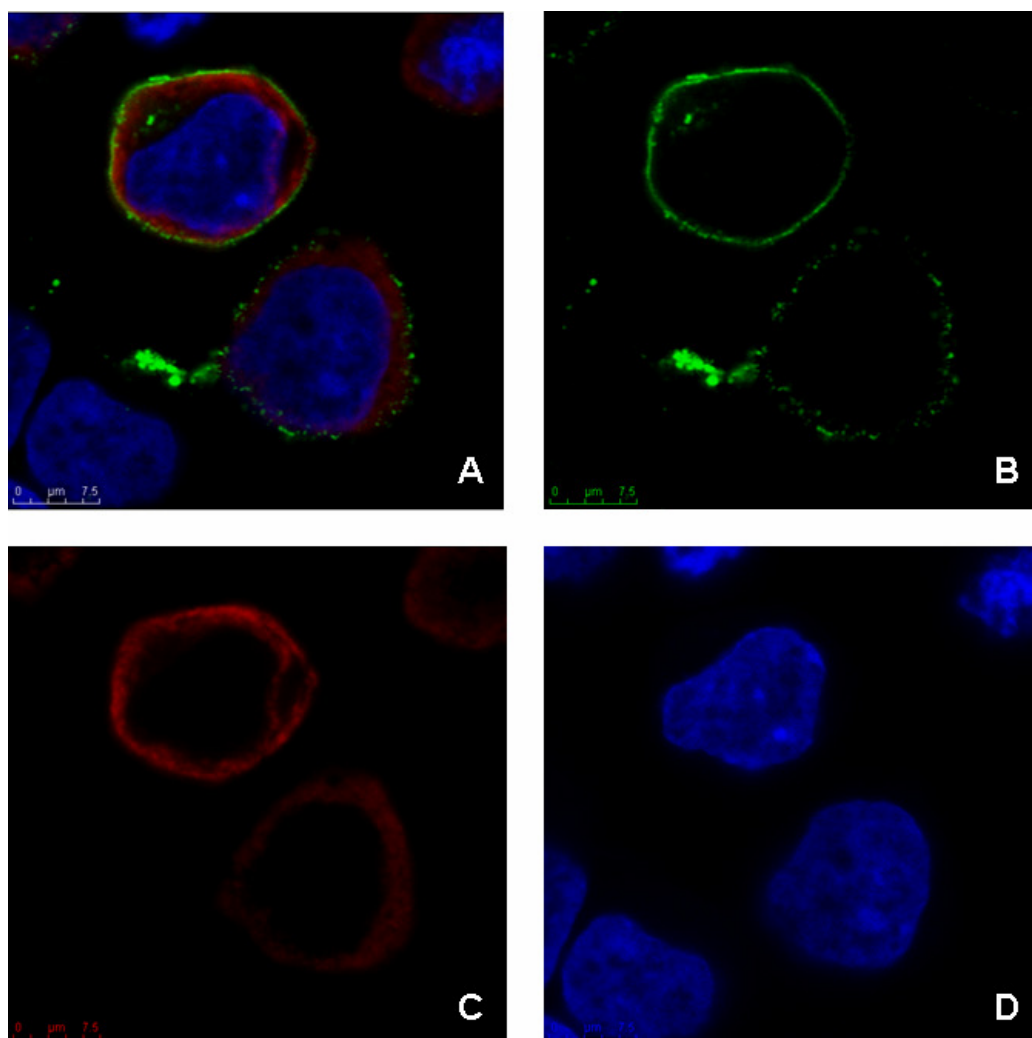


Figure 8.4 Localisation of co-expressed A3G and basic linker mutant Pr55^{GAG} (GM) proteins: 293T cells were co-transfected with plasmids expressing the mutant Pr55^{GAG}-GFP fusion and HA-tagged A3G proteins using the standard lipofectamine transfection protocol detailed in section 2.5.3. 48 hours post-transfection, the cells were processed and analysed as described in section 2.9. GFP fluorescence indicates the position of GM. A3G was detected by using mouse anti-HA as the primary antibody followed by red fluorescent Alexa Fluor 594 goat anti-mouse as the secondary antibody. Nuclei are stained blue with the nuclear stain DAPI. Panel B shows the position of the mutant GM protein. Panel C depicts the position of the red fluorescent HA-tagged A3G and panel D shows the nuclei of cells stained with DAPI. Panel A image is the merge of images B, C and D. A magnification bar of 7.5 μm is present in the bottom left corner of each image.

When Vif was expressed alone in cells, it was distributed diffusely across the cell but with some concentration in the cytoplasm compared to the nucleus (Fig. 8.5 A and B). Thus there was no visible difference found in the localisation of Vif when expressed alone or when in combination with Pr55^{GAG}.

When A3G was expressed alone in cells, it exclusively localised to the cytoplasm (Fig. 8.5 D) with no visible signal in the nucleus (Fig. 8.5 C). Thus the localisation of A3G in cells was similar in both the presence or in the absence of Pr55^{GAG}.

When both G and GM proteins were expressed individually in cells, they both localised to the cytoplasmic membrane (Fig 8.6 A, B and C, D). In a subset of cells, both the G and GM were seen to be diffusely scattered across the cell, mainly in the cytoplasm but a small fraction of them was present in the nucleus as well. This phenomenon was also observed with co-expression of Pr55^{GAG} with Vif/A3G described earlier, where in some cells GAG was seen diffusely spread across the cytoplasm and also in the nucleus to some extent in addition to its strong localisation at the plasma membrane. The reason for this phenomenon could be the cells being at a different stage of GAG protein synthesis and assembly. The cells where GAG is present in the cytoplasm are presumably at an earlier stage than the cells where GAG has reached its ultimate destination, the plasma membrane, for particle assembly. The other possible reason could be due to the over-expression of GAG proteins inside cells which might lead to the swamping of some cells and hence non-specific localisation of Pr55^{GAG} within the cell. However it is important to note that the highest intensity of fluorescent signal obtained with both G as well as GM was still from the plasma membrane in all cells.

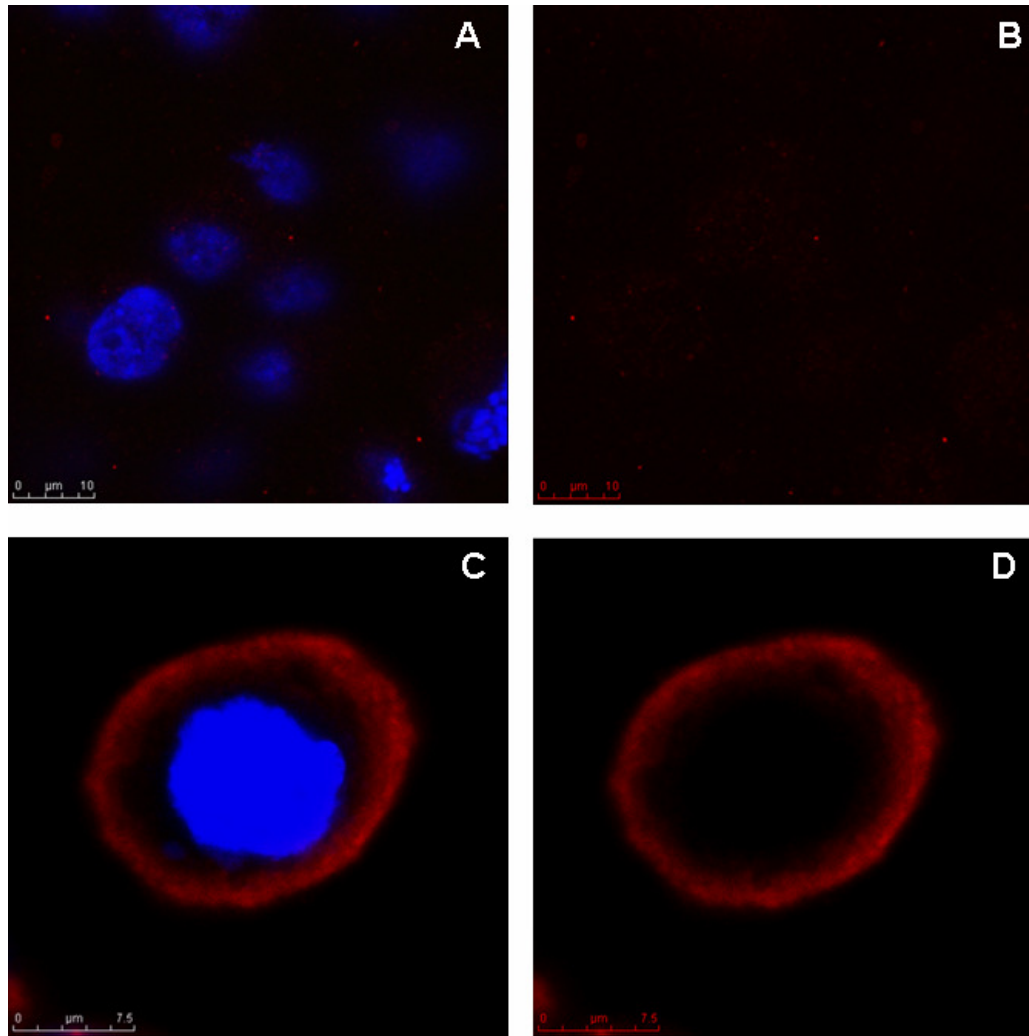


Figure 8.5 Localisation of Vif and A3G proteins expressed singly in cells: 293T cells were individually transfected with plasmids expressing Vif and HA-tagged A3G proteins using the standard lipofectamine transfection protocol detailed in section 2.5.3. 48 hours post-transfection, the cells were processed and analysed as described in section 2.9. Vif was detected by using a Rabbit HIV-1_{HXB2} Vif antiserum as primary antibody followed by red fluorescent Alexa Fluor 594 goat anti-rabbit as the secondary antibody. A3G was detected by using mouse anti-HA as the primary antibody followed by red fluorescent Alexa Fluor 594 goat anti-mouse as the secondary antibody. Nuclei are stained blue with the nuclear stain DAPI. Panels B and D depict the positions of the Vif and A3G proteins respectively. Panels A and C are merged images of B and D respectively with nuclear stain DAPI. A magnification bar of 10 μm is present for images A and B and 7.5 μm for images C and D in the bottom left corner.

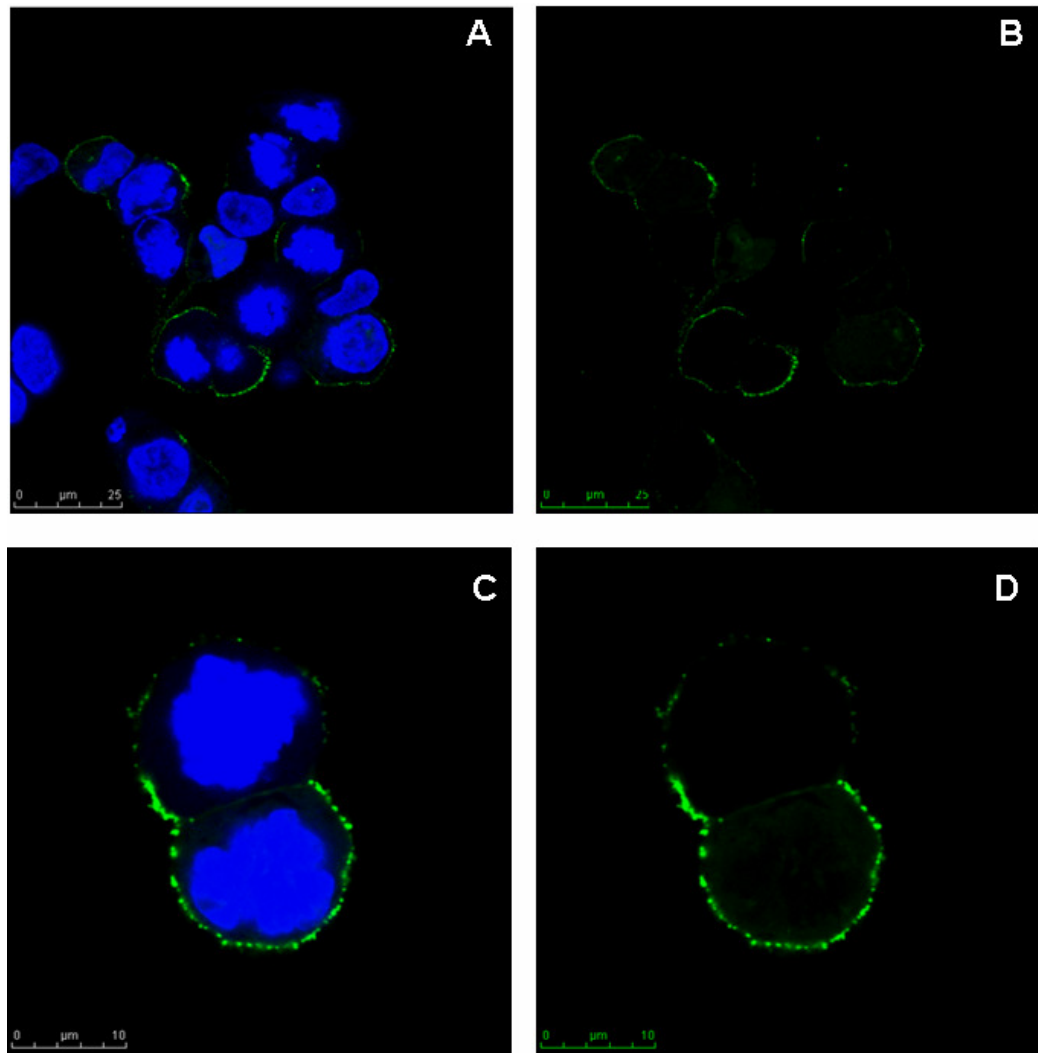


Figure 8.6 Localisation of wild-type (G) and mutant Pr55^{GAG} (GM) proteins: 293T cells were individually transfected with plasmids expressing GFP tagged G and GM proteins using the standard lipofectamine transfection protocol detailed in section 2.5.3. 48 hours post-transfection, the cells were processed and analysed as described in section 2.9. GFP fluorescence indicates the position of the GAG proteins G and GM inside cells. Nuclei are stained blue with the nuclear stain DAPI. Panels B and D depict the positions of G and GM respectively. Panels A and C are merged images of B and D respectively with nuclear stain DAPI. A magnification bar of 25 μm is present for images A and B and 10 μm for images C and D in the bottom left corner of each image.

8.6 Summary

Both A3G and Vif have been shown to interact at the same site on Pr55^{GAG}, in the basic linker region of its nucleocapsid domain (chapter 3). Furthermore, Vif has been shown to quench the signal of the CAT reporter in an assay driven by the A3G-Pr55^{GAG} interaction suggesting that Vif competes with A3G for Pr55^{GAG} binding (chapter 5). The experiments described in this chapter were performed to compare the localisation of both Vif and A3G in the presence of wild-type Pr55^{GAG} (G) or the basic linker GAG mutant (GM).

No visible change was observed in the localisation pattern of either Vif or A3G in the presence of GM when compared to their co-expression with G. However the localisation patterns of Vif and A3G were found to differ considerably from each other. Vif was diffusely scattered across the whole cell, whereas A3G was exclusively compartmentalised to the cytoplasm with no evidence of its presence in the nucleus.

When Vif was co-expressed with either G or GM, there was overlap of a small fraction of Vif with G or GM at the plasma membrane. However the extent of this localisation was small compared with the amounts of each protein expressed. Hence it was not possible on the basis of these data to conclude that there was a specific localisation between Vif and Pr55^{GAG}. In the case of A3G, the overlap of a subset of the protein with either G or GM was much more prominent than that seen with Vif, allowing it to be argued that a fraction of A3G protein was co-localising with both G and GM proteins.

However it was not possible to accurately quantify the extent of this colocalisation. A better experimental design than that employed with 293T cells would have been to infect the non-permissive cells like H9 with wild-type HIV-1 and mutant HIV-1 and then probe the various proteins expressed within cells. This approach would have mimicked the *in vivo* infection model of HIV-1 very closely. However one problem that could have been encountered in this alternative strategy might be the sensitivity of the antibodies used to detect very low levels of endogenous Vif, A3G and Pr55^{GAG} proteins. Of note here is the low sensitivity of the primary antibody used in the detection of Vif in these studies resulting in very low signal intensity.

An additional question that these studies were intended to address was to investigate if there was any change in the localisation pattern of either Vif or A3G in the presence or absence of Pr55^{GAG}. However the experimental controls consisting of Vif, A3G, G and GM proteins expressed individually in cells gave similar localisation patterns compared to those seen when they were co-expressed with other proteins. Thus co-expression with Pr55^{GAG} had no effect on the localisation pattern of either Vif or A3G.

In the light of these results, it becomes important to discuss the proposed model for the competition between Vif and A3G for Pr55^{GAG} binding and virion incorporation. It is known that different GAG molecules in cells meet with different fates (Klein et al, 2007). An immature HIV virion has been estimated to contain ~5000 molecules of Pr55^{GAG} (Briggs et al, 2004). However various reports suggest that the number of Vif/A3G molecules incorporated into virions is only in the region of ~1% of the total GAG molecules (Liu et al, 1995; Xu et al, 2007). Thus not every GAG molecule can be interacting with a Vif/A3G molecule. However it is possible that interaction is

restricted to a few designated GAG molecules which are marked by the presence of some intermediary such as viral RNA, and the competition between Vif and A3G for GAG binding occurs at this intermediary. While Vif has been shown to have a strong affinity for the HIV-1 RNA in both the 5' UTR and GAG region sequences (Bernacchi et al, 2007; Henriët et al, 2005) , there is controversy about the RNA involvement in the packaging of A3G into virions. Whether RNA is involved or not and whether if it is involved, is it cellular 7-SL RNA or viral genomic RNA (Bach et al, 2008; Khan et al, 2007; Khan et al, 2005; Strebel & Khan, 2008; Svarovskaia et al, 2004; Wang et al, 2007a) are all still open to question (section 9.2.2).

Thus it could be argued from the results obtained in this section that it is more likely that the competition between Vif and A3G for Pr55^{GAG} binding requires the presence of an intermediary, and viral genomic RNA seems excellently placed to complete this jigsaw.

CHAPTER 9

Discussion

9.1 General summary

The relative importance of the innate immune response in combating virus infections has become recognised with the increased understanding of the counter-measures that various viruses have evolved to block different facets of this response. The A3G based block to lentivirus replication and the Vif accessory protein that acts to block it is a good example of the type of innate immune response for which the virus has evolved an effective counter-measure. Consequently insights into this mechanism of action may provide for the development of novel anti-viral strategies.

The currently favoured model for Vif's primary mechanism of action involves it promoting the ubiquitination of A3G through stimulating its association with a Cullin 5 (Cul5) based E3 ligase which in turn leads to the proteasome mediated degradation of A3G to lower its intracellular level below the undefined threshold necessary for its incorporation into maturing virus particles (Yu et al, 2003). However the failure of this model to adequately account for an increasing number of observations is prompting its re-evaluation (Ao et al, 2008; Kao et al, 2004; Opi et al, 2007). The early recognition that A3G becomes incorporated into maturing virions through a specific interaction with Pr55^{GAG} coupled with *in vivo* confirmation (Syed & McCrae, 2009) of previous reports *in vitro* (Bouyac et al, 1997a) showing that Vif also interacts specifically with Pr55^{GAG} prompted speculation that an alternative model of Vif's primary action may be operating in which it would compete with A3G for incorporation into the virus particle. The simplest model for such a competition would involve both proteins interacting with the same site on Pr55^{GAG}.

The first observations on the interaction between A3G and Pr55^{GAG} pointed to it binding to the I domain located near the amino terminus of the NC region of the protein (Luo et al, 2004). However this report was contradicted in a subsequent study which indicated that the basic linker region somewhat further along the NC component of Pr55^{GAG} was involved in this interaction (Burnett & Spearman, 2007). The results reported here in which only the substitution mutations (NCSub) in NC affected the interactions are in agreement with these later studies (Chapter 3). However Spearman *et al.* had employed truncated versions of Pr55^{GAG} to study the sites of interaction of A3G with GAG. Hence the experiments conducted as a part of this thesis employing a full length version of Pr55^{GAG} protein are more accurate than any of the previously published reports attempting to analyse the A3G-Pr55^{GAG} interaction. Precise localisation of the interaction sites for the Vif- Pr55^{GAG} interaction has not been reported previously but the results reported here demonstrating that Vif interacts with the same region of NC as A3G provides support for a simple competition model since it allows for the binding of one protein to block the binding of the other (Chapter 3). As neither Vif nor A3G was able to interact with the NC-Sub mutant, it was important to show that this mutated Pr55^{GAG} was still functional and this was done by demonstrating that it could still self-assemble into virus-like particles (Chapter 4).

Having increased the probability of competition between A3G and Vif for incorporation into maturing virus particles, it was important to show that competition between the two proteins for interaction with Pr55^{GAG} occurred *in vivo*. The modification to the two-hybrid assay in which increasing levels of a BD-Vif fusion were able to compete with AD-A3G for interaction with BD- Pr55^{GAG} provided clear evidence that this is the case (Chapter 5). This observation which indicates that Vif

has a higher affinity for Pr55^{GAG} than A3G was confirmed by the finding that increasing levels of AD-A3G were not able to block the interaction between BD-Vif and AD-Pr55^{GAG}. One possible interpretation of this result could be that increasing amounts of BD-Vif was competing with BD-Pr55^{GAG} for the UAS on the reporter vector thereby reducing reporter expression. However this was found not to be the case by employing the empty BD vector, the hypothesis being that if BD-Vif was competing with BD-Pr55^{GAG} for the UAS on the reporter vector, empty BD vector would also exert a similar effect and if this was found to be the case there would be a uniform reduction in reporter expression across all samples when compared to the stepwise reduction seen previously. However increasing the BD-Vif levels in a two-hybrid assay driven by AD-A3G and BD-Pr55^{GAG} and balancing the total amount of DNA with empty BD vector also resulted in a stepwise reduction in reporter gene expression implying that the competition was indeed occurring between Vif and A3G for Pr55^{GAG} binding. It was also important to ensure that these results were not due to the increased levels of BD-Vif leading to the proteasome based degradation of AD-A3G and hence lower levels of reporter gene expression in the two-hybrid assay. The fact that the levels of HA tagged A3G expressed in co-transfected cells remained broadly constant across the range of BD-Vif used showed that it was not promoting the degradation of A3G under the assay conditions employed (Chapter 5).

To independently corroborate these competition data, sucrose gradient analysis of GAG VLPs was performed (Chapter 6 & 7). However this strategy didn't yield the desired results due to the failure of the positive control to function optimally. The most probable cause of this failure could be due to the non-specific incorporation of both Vif and A3G into GAG virus-like particles. As all the proteins in this study were over-expressed in cells, the high levels of Vif or A3G could have then bound

non-specifically to Pr55^{GAG} to get incorporated into the budding GAG VLPs. This made it very difficult to measure the specific incorporation of these proteins in VLPs in turn making it impossible to accurately analyse the data obtained from these experiments.

When the localisation patterns of Vif, A3G, Pr55^{GAG} and the basic linker GAG mutant were studied using confocal imaging, no visible change in localisation of either Vif or A3G was observed when they were co-expressed with the GAG mutant when compared to their co-expression with wild type Pr55^{GAG}. Also no change was detected in the localisation patterns of both Vif/A3G when expressed individually or in combination with Pr55^{GAG} (Chapter 8). These results when analysed together with the stoichiometry of both these proteins in the budding HIV virion point towards a scenario where the competition between Vif and A3G could be occurring at a few designated GAG molecules, the molecules being determined by the presence of an intermediary, possibly viral genomic RNA, which mediates these interactions.

The finding of competition *in vivo* between Vif and A3G for interaction with Pr55^{GAG} allows for the proposition of an alternative model for the action of Vif in which it overcomes the A3G based inhibition of virus replication by blocking its incorporation into the assembling virus particle. This action of Vif being assisted by its well characterised ability to lower the levels of A3G present in infected cells by promoting its ubiquitination and subsequent targeting to the proteasome for degradation.

In this new model of Vif action the main observation that remains to be understood is how this competition can be achieved in a way that is consistent with the known stoichiometry of these proteins in the assembled virion. There are various estimates

of the amount of Vif or A3G found in mature virus particles, but none of these amounts to more than a small fraction (~1%) of the number of Pr55^{GAG} molecules involved in forming a mature virion (Liu et al, 1995; Xu et al, 2007). The following could be the probable scenarios where the proposed new model for Vif's primary action could fit within the parameters of existing data available regarding the stoichiometry of the interacting components within mature HIV virions:

- (a) The first possibility which seems more likely considering the evidence in its favour is the involvement of the same RNA intermediary for the interaction of both Vif and A3G with Pr55^{GAG}.
- (b) The second possible explanation would be a direct interaction between Vif and Pr55^{GAG} such that every GAG molecule in the cell is bound by Vif thereby blocking A3G binding to Pr55^{GAG} and thus preventing its incorporation into budding virus particles. This looks unlikely because of the immunofluorescence data obtained where not every Pr55^{GAG} molecule was seen to co-localise with co-expressed Vif/A3G.

Both these possibilities have been addressed in greater detail in section 9.2.2 later in this chapter. Clearly further studies will be needed to provide an explanation of how the proposed new model for Vif's primary action fits with the stoichiometry of the interacting components.

9.2 Future work

9.2.1 Validating the conclusions drawn from biochemical assays in this study with whole virus based infectivity assays:

All the results in this study have been obtained from surrogate assay systems due to the practical constraints placed on working with infectious HIV. Two important limitations of such assays are that they only involve a subset of the HIV gene complement and as biochemical assays they lack the dynamic range available in biological infectivity assays. Hence the immediate logical step forward would be to validate the findings obtained in the mammalian two-hybrid assays with whole virus based infectivity assays.

Hence the aim would be to design virus infectivity assays where the following questions would be answered:

9.2.1.1 Do Vif and A3G interact at the same site on Pr55^{GAG}?

The NCSub mutant of Pr55^{GAG} which resulted in a huge drop in CAT reporter expression with both Vif as well as A3G had two substitution mutations in its Pr55^{GAG} sequence whereby two basic amino acids namely Arginine and Lysine in the basic linker region of the NC component of the precursor were replaced by two acidic amino acids namely two Glutamic acids. These same mutations could be possibly incorporated into the Pr55^{GAG} gene sequence in an infectious molecular clone of HIV, although of course it must be acknowledged that such a viral mutant may not be viable.

The hypothesis would be that any virus carrying these NC substitutions would not be able to incorporate either Vif or A3G within its budding particles. Theoretically, this would mean that the virus would be able to produce fully infectious virus from non-permissive cells which constitutively express A3G as efficiently as permissive cells, even in the absence of a functional Vif protein.

However this would in effect mean that the resultant mutated virus would be potentially more virulent than wild-type HIV-1. Hence it would be prudent to restrict the host range of the mutant carrying such mutations by for example incorporating deletions in the env gene such that growth would be restricted to cells in which complementing env gene product was being expressed.

9.2.1.2 To study the competition between Vif and A3G for Pr55^{GAG} binding:

The aim would be essentially to repeat the experiments carried out in Chapter 6, with the only difference being that an infectious clone of HIV would be employed instead of a Pr55^{GAG} expression plasmid.

The experiment would entail measuring the infectious viral yield obtained when an infectious molecular clone of HIV-1 is allowed to co-express with the degradation-resistant variant of A3G. The hypothesis would be that inspite of not being able to degrade the variant, the Vif protein in HIV will still be able to outcompete A3G and prevent its incorporation into newly formed progeny virions thereby preserving the normal viral infectivity. Thus the reduction observed in the level of the A3G variant in the progeny virions in presence of a functional Vif protein would be roughly similar to the decrease in the amount of wild-type A3G observed in virions.

However, there could still be a potential problem of non-specific incorporation of transiently expressed A3G. The more technically correct experimental set-up would

mean studying viral infectivity in a cell line which stably expresses the degradation resistant variant of A3G instead of transiently expressed protein as this set-up would mimic closely the natural course of infection of the virus in non-permissive cells constitutively expressing A3G.

9.2.2 Does a requirement for RNA explain the stoichiometry of the Vif-Pr55^{GAG} and A3G-Pr55^{GAG} interaction?

While the competition hypothesis of Vif action has been demonstrated in the mammalian two-hybrid data in this thesis, it is puzzling that only ~1% of the Pr55^{GAG} assembled into the maturing virion appears to be interacting with either Vif or A3G. One way to account for this is to propose that the interactions between these proteins requires a third component, specifically viral RNA, and it is the interaction with this third component at a critical time in the virion assembly process that both governs the competition between Vif and A3G and determines the amount of either protein finally encapsidated into the infectious virion. The overall stoichiometry of the process being determined by saturation of the RNA sequence/structure required for Pr55^{GAG} –Vif/A3G interaction with one of the interacting proteins, which *in vitro* studies have pointed to being Pr55^{GAG} (Zhang et al, 2000). The area of RNA involvement in Vif and/or A3G packaging is currently one of considerable controversy, with no clear consensus in the literature concerning the involvement of particular RNA species in the packaging of either protein or indeed general agreement as to whether any RNA is involved in the interaction between these proteins.

Vif is a basic protein at physiological pH which has intrinsic nucleic acid binding activity, but the consensus view is that it has a particular affinity for HIV RNA

(Dettenhofer et al, 2000; Khan et al, 2001) and more specifically for the 5'UTR and GAG region of the viral genome (Bernacchi et al, 2007; Henriët et al, 2005). Interestingly, sequences covering the region of GAG known to be involved in the Vif-RNA interaction are present in the Pr55^{GAG} plasmid constructs used in two-hybrid interaction assays carried out in this study (Chapter 3). Hence it is possible that the positive interaction seen between Vif and Pr55^{GAG} is due to the presence of the RNA intermediary corresponding to the region of GAG involved in the Vif-RNA interaction.

The picture for A3G is even more complex with no agreement as to whether RNA is required for its packaging and further disagreement as to whether it is cellular 7SL RNA or viral RNA that is involved (Bach et al, 2008; Khan et al, 2007; Khan et al, 2005; Strebel & Khan, 2008; Svarovskaia et al, 2004; Wang et al, 2007a).

To investigate the involvement of RNA in the interaction between Pr55^{GAG} and Vif or A3G two *in vitro* assays can be used.

9.2.2.1 Qualitative assay

The expression of GST- Pr55^{GAG} in E.coli and its purification for use in *in vitro* assays has been accomplished successfully at Warwick (Syed, 2008). Expression constructs for Vif and A3G have also been made for producing radio-labelled proteins in a T7 based coupled transcription-translation system from which all endogenous mRNA has been removed post-translationally using micrococcal nuclease treatment (Pelham & Jackson, 1976). Using these reagents, assays could be set up to (a) investigate the ability of the GST-Pr55^{GAG} to retain radio-labelled Vif or A3G on glutathione-sepharose beads, (b) analyse the involvement of specific RNAs in this interaction and (c) decipher the order in which these entities need to be mixed

in order to facilitate retention. This assay could shed light on the qualitative requirement of specific RNAs required for the interaction between these components.

9.2.2.2 Quantitative assay

To obtain information on the relative affinities of Vif or A3G for Pr55^{GAG} and the effect(s) of specific RNAs on such affinities, surface plasmon resonance (SPR) based assays in a Biacore using chips coated with immobilised GST-Pr55^{GAG} fusion protein can be employed (Nakadai et al, 2004; Nakajima et al, 2005). In-vitro expression of Vif/A3G is unlikely to produce sufficient protein to be used as the analyte in these assays. So bacterially expressed and purified GST-Vif and GST-A3G constructs can be used from which if necessary the GST tag can be removed using Factor Xa cleavage (Syed, 2008). This assay would give quantitative information regarding the relative affinities of Vif and A3G for Pr55^{GAG} thus providing indirect supporting evidence towards the competition hypothesis of Vif action. In addition, it can also give an insight into the involvement of specific RNAs in the Vif/A3G- Pr55^{GAG} interaction.

The aim of both of these experimental approaches will be to assess if and how RNA is involved in the interaction between Pr55^{GAG} and Vif/A3G and to confirm the nature and location of the sequences/structures necessary for these interactions through supplementing the assays with specific RNAs generated from transcription vectors. The weight of current evidence does suggest that an RNA intermediary is involved in the interaction process. If this is confirmed to be the case then it could explain how competition between Vif and A3G occurs in light of the stoichiometry of the final amounts of these proteins in the virus particle.

It does of course still remain possible that this will not prove to be the explanation, in which case an alternative possibility can be investigated. The molar amounts of Pr55^{GAG} and Vif synthesised in virus infected cells are broadly similar and so it remains possible that every molecule of Pr55^{GAG} is involved in an interaction with Vif shortly after its synthesis, blocking any possible interaction with A3G. If this occurred then some novel mechanism would be required to break the interaction at the cell membrane as Pr55^{GAG} is being assembled into virus particles and thereby strip the great majority of the Pr55^{GAG} molecules of their interacting Vif. This model of Vif action also demands that none of the A3G present in virus infected cells would be able to access Pr55^{GAG} at the cell membrane as molecules stripped of their interacting Vif were generated. This hypothesis could be investigated using co-immunoprecipitation studies which would predict that early in the replication cycle prior to virus particle formation, exhaustive immunoprecipitation with anti-Vif sera would remove all Pr55^{GAG} from cell lysates meaning that subsequent precipitation of the lysate with anti-p24 sera would fail to precipitate any Pr55^{GAG}.

9.2.3 What is the minimum region of Pr55^{GAG} required for interaction with Vif or A3G?

If competition for interaction with Pr55^{GAG} proves to be the primary mechanism by which Vif functions to overcome the A3G based inhibition, then a novel approach to antiviral development could be to titrate out the Vif in infected cells by having it bind to a peptide analogue of Pr55^{GAG}. This would leave the A3G expressed in the host cells to interact with viral Pr55^{GAG} and hence get incorporated into assembling virions with its consequent inhibitory effects on subsequent virus replication. For this approach to be viable the interaction site for Vif and A3G on Pr55^{GAG} would need to

be sufficiently discrete that it can be mimicked in a small peptide capable of being efficiently delivered to cells. Our studies on the interaction site have shown that it involves the basic linker region of the NC component of Pr55^{GAG}. Subsections of Pr55^{GAG}, starting with full length NC (~70 amino acids), can be assayed in the mammalian two-hybrid system for their ability to interact with Vif/A3G. If full length NC gives positive interaction with Vif and A3G then smaller fragments of NC could be used in these experiments with the objective of localising the minimum size peptide necessary for positive interaction with Vif. If evidence from experiments in section 9.2.2 points towards the involvement of viral genomic RNA in the interaction of both Vif and A3G with Pr55^{GAG} then co-expression of HIV RNA covering more 5' terminal regions of the HIV genome may be needed in these experiments to achieve a positive interaction. After defining the minimum Pr55^{GAG} peptide needed for the Vif interaction, the next step would be to examine the feasibility of using peptide transfection of virus infected non-permissive cells as a route to blocking the action of Vif and hence developing a novel approach to anti-retroviral therapy.

To date, we are a long way away from finding a satisfactory cure to HIV/AIDS or even from the development of an efficacious vaccine against this virus. Hence the need of the hour is to study the life-cycle and mechanism of replication of HIV on a molecular level and identify new targets which could be potentially used to develop novel anti-retroviral drugs.

Towards the same, the findings in this study are a step forward in this direction. Although the research results in this study warrant further detailed analyses, these findings have the potential to make a significant impact to the field. Importantly, this new hypothesis provides a testable system for the rational development of novel antiviral strategies that target Vif function.

APPENDIX

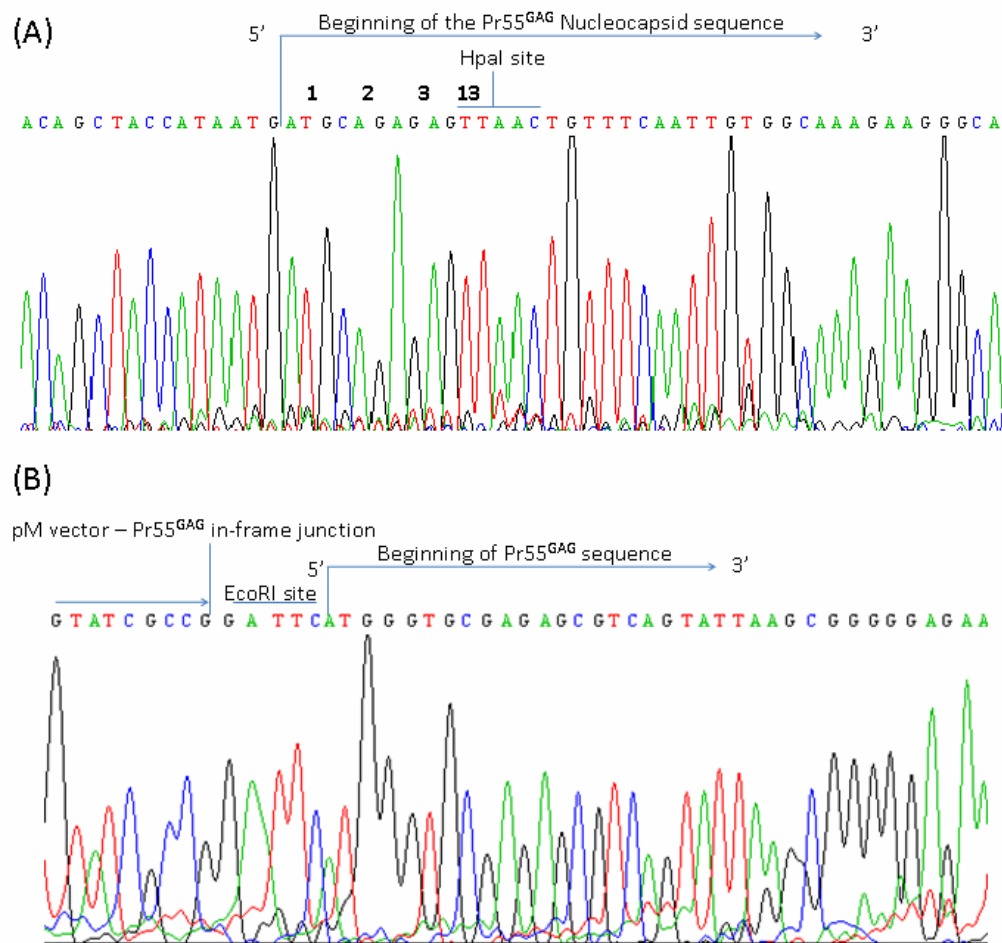


Figure 1.1 Sequence analysis of the NCDel mutant of Pr55^{GAG}. (A) Chromatogram showing the deletion of amino acids 4 to 12 from the N-terminal region of the Nucleocapsid domain of Pr55^{GAG}. The beginning of the Nucleocapsid sequence has been indicated by arrows and the amino acid number has been highlighted alongside the position of the HpaI site. (B) Chromatogram showing the in-frame junction of Pr55^{GAG} with pM vector DNA binding domain (BD) separated by an EcoRI site in the NCDel mutant of Pr55^{GAG}.

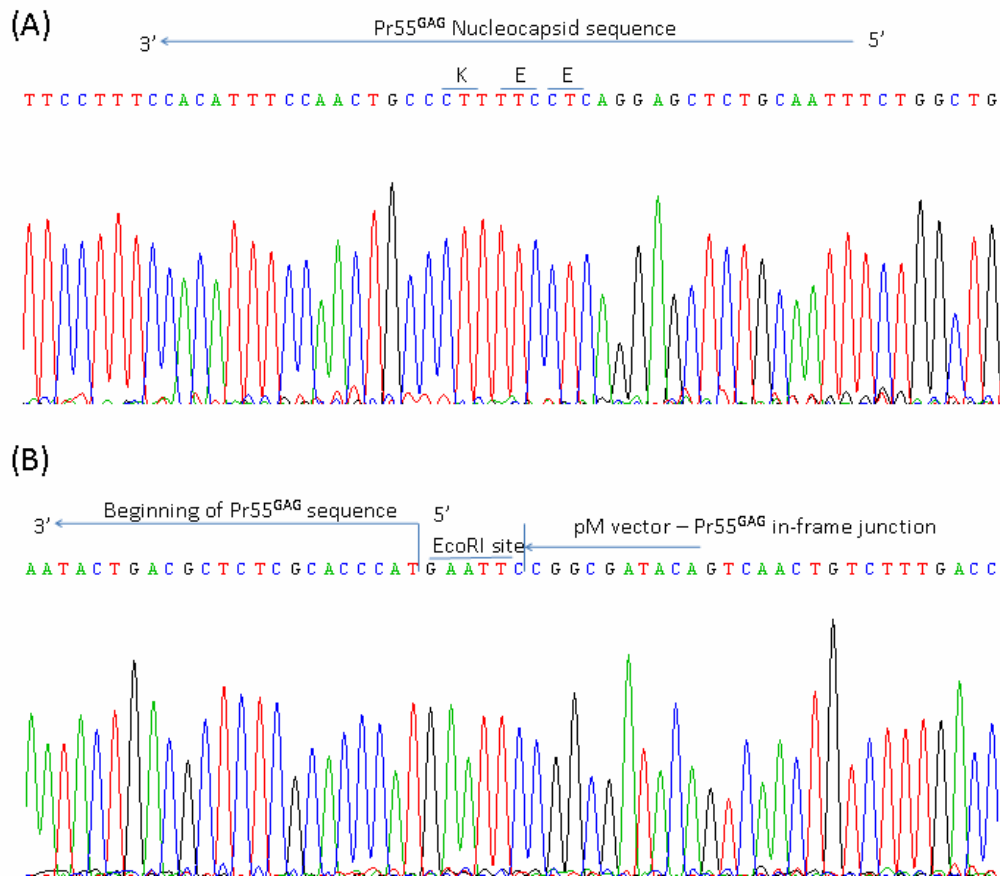


Figure 1.2 Sequence analysis of the NCSUB mutant of Pr55^{GAG}. (A) Chromatogram showing the substitution of amino acids Arginine (R) and Lysine (K) with two Glutamic acids (E) in the basic linker region of the Nucleocapsid domain of Pr55^{GAG}. The orientation of the Pr55^{GAG} sequence has been indicated by an arrow and the respective amino acid position has been depicted with symbols. (B) Chromatogram showing the in-frame junction of Pr55^{GAG} with pM vector DNA binding domain (BD) separated by an EcoRI site in the NCSUB mutant of Pr55^{GAG}.

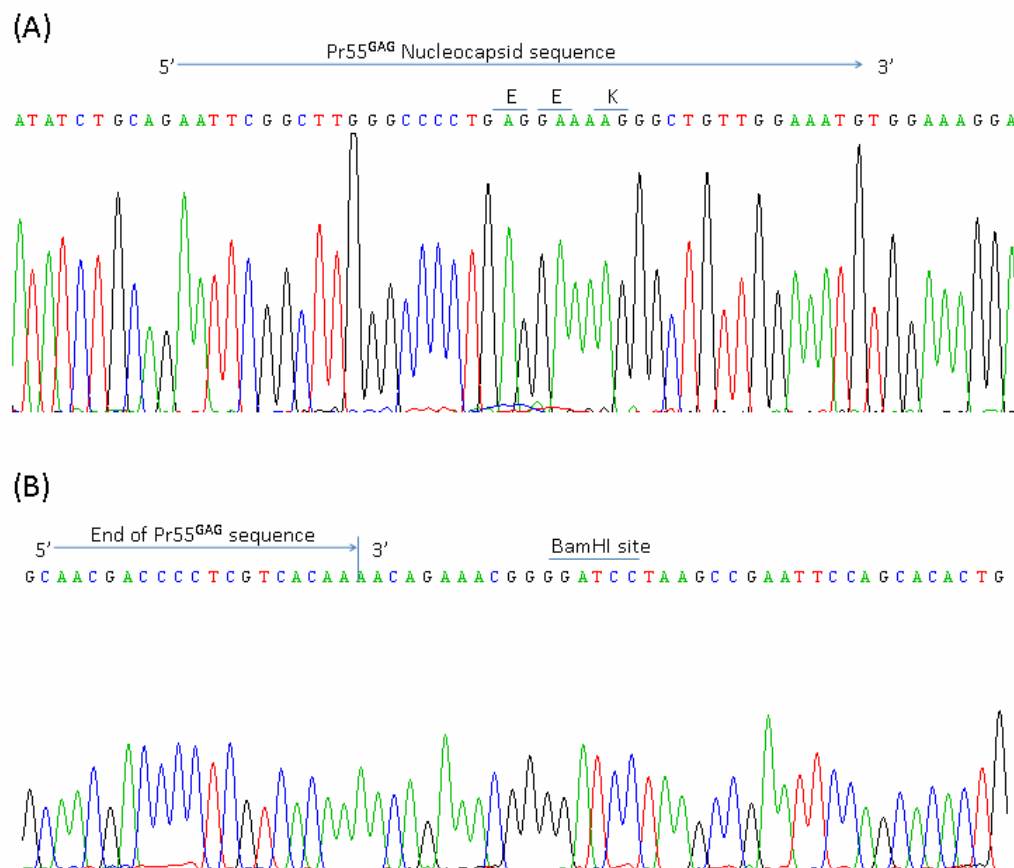


Figure 1.3 Sequence analysis of the GM mutant of Pr55^{GAG}. (A) Chromatogram showing the substitution of amino acids Arginine (R) and Lysine (K) with two Glutamic acids (E) in the Basic Linker region of the Nucleocapsid domain of Pr55^{GAG}. The orientation of the Pr55^{GAG} sequence has been indicated by an arrow and the respective amino acid position has been depicted with symbols. (B) Chromatogram showing the end of the Pr55^{GAG} sequence flanked by a BamHI site in the GM mutant of Pr55^{GAG}.

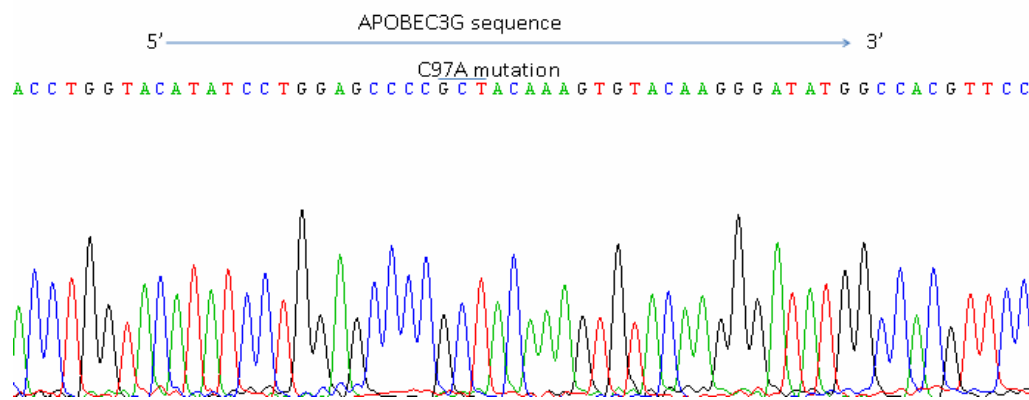


Figure 1.4 Sequence analysis of the degradation resistant variant of A3G (AM). Chromatogram showing the substitution of amino acid Cysteine (C) with Alanine (A) at position 97 within the A3G sequence by site-directed mutagenesis of pcDNA3.1-A3G-HA (A). The orientation of the APOBEC3G sequence has been indicated by an arrow and the position of the mutation has been depicted.

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